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14. **ABSTRACT**
The objective is to design, build and study vectors which would be able to break tolerance to breast cancer associated TAA and be used to suppress the recurrence of metastatic breast cancer following surgical resection. The hypothesis is that by fusing the CD40 ligand stripped of its transmembrane domain and intracytoplasmic domains, to a breast cancer TAA such as the extracellular domain of the her-2-neu recelptor, or the extracellular tandem repeat peptides of breast cancer associated surface glycoporotein, MUC-1 (both of which have been shown to be capable when loaded on APCs of conferring resistance to engraftment by cancer cells bearing these TAA), one can break tolerance to breast cancer. The subcutaneous injection of this vector creates infected cells as factories to secrete the CD40LTAA into the systemic circulation as well as locally for the activation and antigen loading of APCs, so that they would move to the lymph nodes all over the body to generate a CD8 dependent response against metastatic breast cancer. We also explored boosting of the vector vaccine by TAA/CD40L protein injections. This report summarizes the successful assemby and study of these vectors. These injections break tolerance to tumor associated antigens in mouse models.

15. SUBJECT TERMS
Adenoviral vector, gene therapy, metastatic breast cancer, HER2-Neu, MUC1

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Summary Review of Annual Report:

Background and Rationale: The immune response becomes diminished in age groups in which most epithelial cancers occur due to decreases in the number of naïve CD8 T cells and the acquisition of both quantitative and functional defects in CD4 cells. Early in life, the immune response becomes tolerant to most of the tumor associated antigens (TAA) because they are present on normal cells from birth. Even when TAA are specifically present in the cancer cells and not present on normal cells, these tumor specific tumor associated antigens are seldom taken up by antigen presenting cells or dendritic cells (DCs) and when this occurs, they are presented on Class II MHC. Our laboratory has designed an adenoviral vector (Ad-sig-TAA/ecdCD40L) vaccine which is designed for the in vivo TAA loading and activation of DCs so as to overcome the above problems in the immune response in older cancer patients. The subcutaneous (SC) injection of this vector leads to the release of a fusion protein composed of a TAA linked to the extracellular domain (ecd) of the CD40L. This vaccine has been shown to overcome anergy in TAA.Tg transgenic mouse models, and to induce TAA specific memory cells.

Progress in the Past Year: Because of the limited success of vaccines in older individuals and the fact that the Ad-sig-TAA/ecdCD40L vaccine is CD4 independent and therefore potentially not limited by the functional CD4 defects in older people, we carried out vaccination of 18 month old aged tumor bearing mice. These experiments showed an increase in the levels of TAA specific CD8 effector cells in tumor tissue of older mice and a decrease in the levels of the CD4 FOXP3 negative regulatory T cells in the tumor tissue of older mice. In addition, the vaccination in older mice (vector prime/protein boost) induced complete regressions of existing tumor. We also tested the ability of inducing an immune response against markers unique to tumor vascular endothelial cells. This was success as measured by antibodies specific for the tumor vascular endothelial cells in vaccinated mice, and regressions of tumor in the vaccinated mice.

TASK Report:

Tasks 1-4 in progress.

Body of Report.

I. Introduction.

This is a report of the results of the research for the period of July 14, 2004-July 14, 2005. The goal of the studies was to test the effect of subcutaneous injections of TAA/ecdCDr40L vectors and proteins in mice which were anergic to the TAA. The ultimate goal of this research is to develop a clinical vaccine protein for breast cancer patients who are at high risk of recurrence.

II. Body.

II.A. Approach.

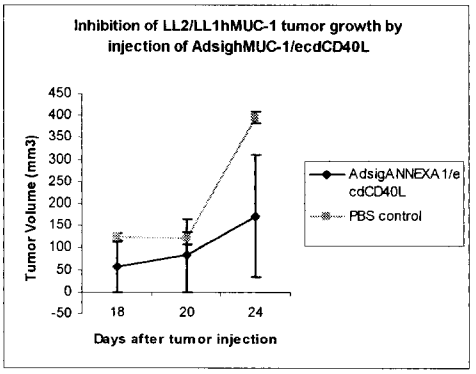
II.B. Overview of Summary of Results.

II.C. Detailed Presentations of Results.

II.C.1. Activation of Immune Response Against Antigens Unique to the Tumor Vascular Endothelial Cells.

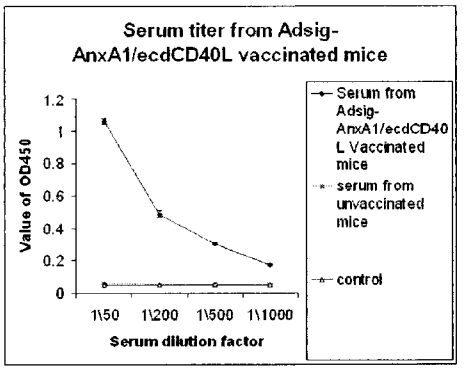
II.C.1.a. The Ad-sig-AnxA1/ecdCD40L Induces an Immune Response Against Antigens Unique to Tumor Vascular Endothelial Cells (TVECA). Schnitzer and co-workers recently reported (1-2) that the Annexin 1A (Anx1A) protein is present on the luminal surface of the endothelial cells of tumor vasculature but was not detectable on the luminal surface of the vascular endothelial cells of normal tissues. We therefore decided to test if the subcutaneous injection of the Ad-sig-Anx1A/ecdCD40L vector would suppress the growth of the hMUC-1 positive LL2/LL1hMUC-1 cell line. As shown below in Figure 1A, the sc injection of this vector suppressed the growth of the hMUC-1 positive cells in hMUC-1.Tg mice.

Figure 1A

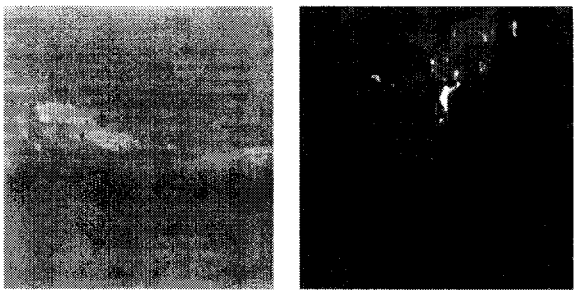


In order to test specifically if the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 antigen, serum was taken from a mouse which had been injected sc twice with the Ad-sig-AnxA1/ecdCD40L vector and tested by ELISA assay for the presence of antibodies against the Annexin A1 antigen.

Figure 1B



As shown in Figure 1B, antibodies which bind Annexin A1 are induced in the serum of the Ad-sig-AnxA1/ecdCD40Lvaccinated hMUC-1.Tg mice. In order to directly test if these antibodies are binding to the tumor vasculature, multiparameter fluorescence confocal microscopy was carried out on frozen sections of hMUC-1 positive, Annexin A1 negative tumor tissue.



Unvaccinated mice Group Vaccinated mice Group

Double staining the tumor tissue with serum from vaccinated mice (green) and PE-labeled Anti-mouse CD31 (red)

Figure 1C

As shown in Figure 1C, the binding of the FITC conjugated (green staining) serum antibodies against Annexin A1 generated in the mice injected sc with the Ad-sig-AnxA1/ecdCD40L vector bind to the tumor vasculature as shown by the yellow spots in the right hand panel in Figure 1C. The red color of the anti-CD31 vascular binding antibody (phycoerythrin conjugated) coincides with the binding of the

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FITC-conjugated serum (stains tissue green) from the Ad-sig-AnxA1/ecdCD40L vaccinated mouse. No yellow color (or green color) appears in the left hand panel of Figure 1C in which frozen sections of tumor tissue was exposed to FITC conjugated serum from unvaccinated mice and the phycoerythrin conjugated anti-CD31 antibodies. These results suggest that the Ad-sig-TAA/ecdCD40L vaccine strategy can induce an immune response against tumor vascular endothelial cells, thereby suppressing the growth of the tumor tissue which depended on the Anx1A positive tumor vasculature.

II.C.1.b. Specificity of the Ad-sig-AnxA1/ecdCD40L Vector Vaccination for Tumor Vascular Endothelial Cells. Annexin A1 is a cytosolic protein in normal ciliated tissues, the central nervous system and in endothelial cells. It is involved in the inflammatory response as well. Therefore, to evaluate the feasibility of using the Ad-sig-AnxA1/ecdCD40L immunization, it was important to test the selectivity of the humoral immune response induced by the Ad-sig-AnxA1/ecdCD40L vaccination. This would in part be dependent on the distribution of Annexin A1 which is intracellular in normal cells but may be available to the extracellular environment in endothelial cells in neoplastic tissue. We therefore tested the binding of serum from the bloodstream of Ad-sig-AnxA1/ecdCD40L vaccinated mice to paraffin embedded formalin fixed sections of tumor tissue (Figure 2A), normal lung-a ciliated tissue (Figure 2B), normal central nervous system (Figure 2C) and normal kidney (Figure 2D). As shown by the brown color encircling the vessel in Figure 2(A), HRP conjugated secondary anti-mouse antibodies produced positive staining in the vessels of tumor tissue, but not around the vessels of normal lung, liver, brain, or kidney (see Figures 2 B-E).

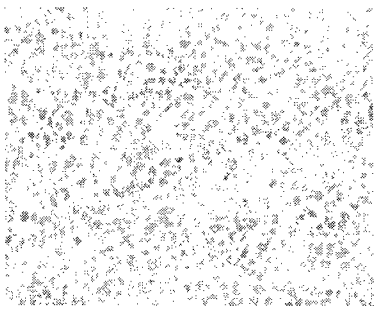
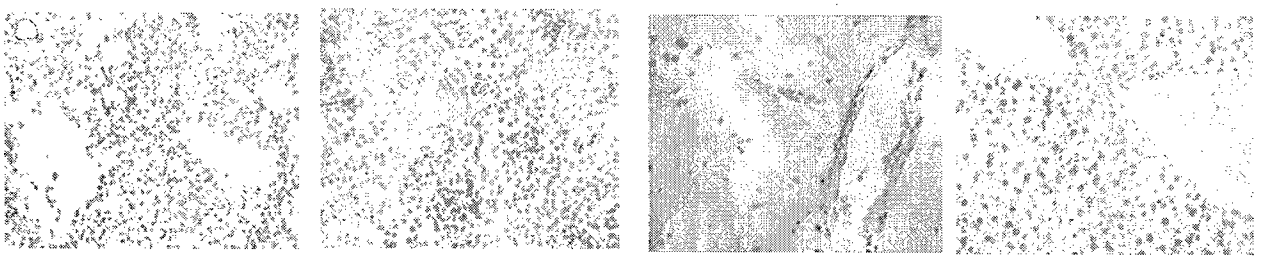


Figure 2A-Tumor

Figure 2B-Lung **Figure 2C-Kidney** **Figure 2D-Brain** **Figure 2E-Liver**



II.C.1.c. Study of the Combination of the Ad-sig-TAA/ecdCD40L and Ad-sig-TVECA/ecdCD40L Vaccines.

In order to test if the combination of the Ad-sig-TAA/ecdCD40L anti-cancer cell vaccine with the Ad-sig-TVECA/ecdCD40L anti-tumor vascular endothelial cell vaccine would produce a tumor suppressive effect that is greater than either vaccine alone, we vaccinated rH2N.Tg mice subcutaneously with the Ad-sig-rH2N/ecdCD40L anti-Her-2-Neu breast cancer cell vaccine and with the Ad-sig-AnxA1/ecdCD40L anti-tumor vascular endothelial cell vaccine. It is noteworthy that the rH2N positive breast cancer cells injected subcutaneously in the vaccinated mice were AnxA1 negative. As shown in Figure 18A, the effect of the combination of the Ad-sig-rH2N/ecdCD40L and the Ad-sig-AnxA1/ecdCD40L vaccines (open squares) was greater than the effect of either vaccine alone (open diamonds or open circles)

We also followed the vaccinated mice following challenge with the rH2N positive breast cancer syngeneic cell line. As shown in Figure 3B, the mice vaccinated with a combination of the Ad-sig-rH2N/ecdCD40L and the Ad-sig-AnxA1/ecdCD40L tumor vascular targeting vaccine vector (open squares) showed the highest percentage of mice which remained tumor free.

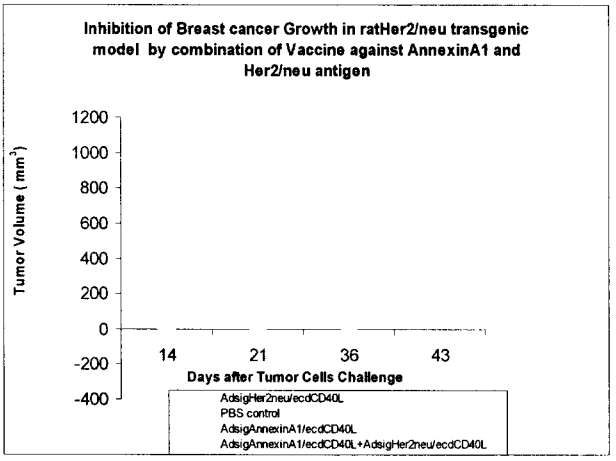


Figure 3A

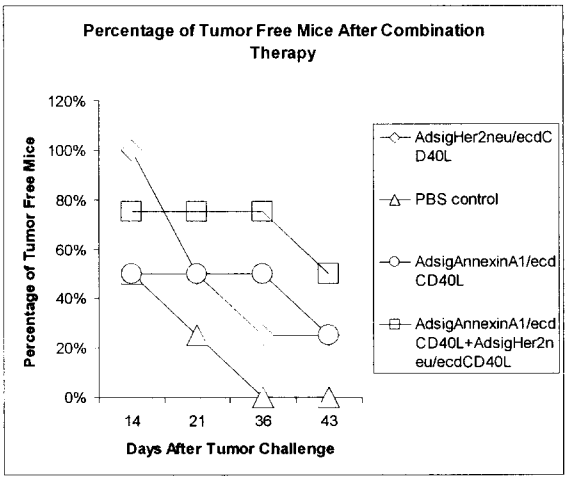
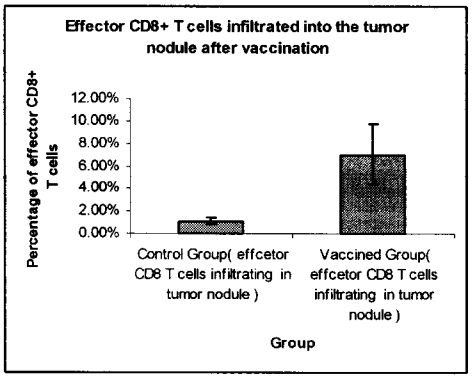


Figure 3B

II.C.2. Levels of CD8 Effector T Cells in Tumor Tissue Following Ad-sig-TAA/ecdCD40L Vector Vaccination of Young Mice. We had shown previously that the SC injection of the Ad-sig-TAA/ecdCD40L vector activated and tumor antigen loaded DCs which then migrated to the regional lymph nodes where the DCs resulted in an increase in the levels of the TAA specific CD8 T cells. One question left unresolved by these earlier studies was whether these TAA specific effector CD8 T cells reached the tumor tissue in the extravascular space. In order to test if the levels of CD8 effector T cells in the tumor tissue would be increased following vaccination, we minced the SC tumor nodules of young (2 months old) rH2N.Tg mice before and after two SC injections of the Ad-sig-rH2N/ecdCD40L vector. Single cell suspensions were generated from the tumor tissue after mincing and treatment with 0.03% DNAse I and 0.14% collagenase I, and filtration through Nylon mesh. We found that the percentage of CD8 T cells with the immunophenotype of effector T cells (CD8+, CD44+, LY6C+and CD62L-) was increased in the tumor tissue after vaccination (see Figure 4). This suggests that the suppression of the growth of the rH2N positive tumor cells in the rH2N.Tg mice following Ad-sig-rH2N/ecdCD40L vaccination is mediated in part by an increase in the trafficking of effector T cells into the tumor tissue.

Figure 4



RNA was isolated from these tumor infiltrating CD8 effector T cells and the pattern of gene expression was compared before and after vaccination using the Affymetrix gene expression system. We also examined the expression level of the 21 known chemokine receptors and ligands in the effector T cells which were infiltrating the tumor tissue. The level of mRNA transcript encoding the CCL3 (2.8 fold increase) and CCR5 (16 fold increase) chemokines were increased in the CD8 effector T cells in the tumor tissue after vaccination. These two chemokines are involved in the targeting of T cells to the extravascular sites of tissue inflammation. The chemokine pathway plays a major role in the trafficking of effector and memory T cells from the lymph nodes draining sites of vaccination or infection to the tissue sites harboring inflammation or infection (3-4).

II.C.3. Study of the Effect of the Ad-sig-TAA/ecdCD40L Vector Vaccination in Old (18 Months) Mice.

II.C.3.a. Changes in Number and Function of TAA Specific CD8 Effector Cells in Tumor Tissue of Old Mice After Vaccination. We chose the HPV E7 antigen for the initial vaccination for the initial studies in the old mice because it was a foreign antigen and had the greatest likelihood of producing a positive response. The first test of the Ad-sig-TAA/ecdCD40L strategy was to vaccinate C57Bl/6J mice which were 18 months old with the Ad-sig-E7/ecdCD40L vector followed in 7 days by a single E7/ecdCD40L protein boost SC injection. Seven days later, we sacrificed the mice and then measured the level of the E7 specific T cells in the spleen by ~~ELISPOT assay (see below in Figure 5A). As shown in Figure 5A, the levels of E7 specific interferon gamma~~

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positive T cells in the spleen of old mice was increased to 120 antigen specific T cells/100,000 CD8 Tcells by vaccination. As shown in Figure 5B, when we vaccinated 18 month old tumor bearing mice with one vector SC injection followed by 3 SC E7/ecdCD40L protein injections, the level of interferon-gamma positivecells positive cells/100,000 splenocytes was increased to 250 in the old mice.

Figure 5A

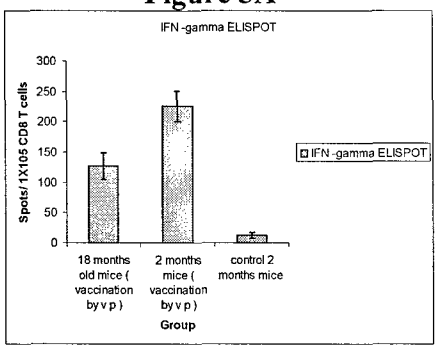


Figure 5B

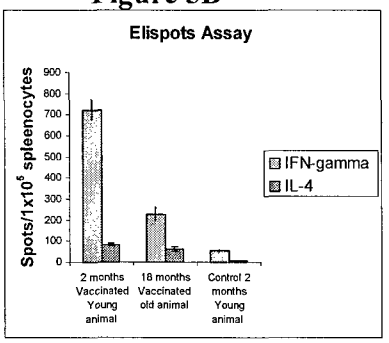
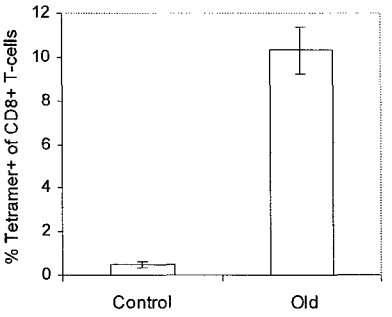


Figure 6A



We then measured the increase of the percentage that antigen specific T cells constituted of total CD8 T cells in the tumor tissue before and after vaccination using E7 tetramers. As shown in Figure 6A, the Ad-sig-E1/ecdCD40L vaccine induced the level of antigen specific T cells in the tumor tissue by 10 fold. We also measured the increase of the T cells as a percentage of the total number cells in the tumor tissue following vaccination in the old mice. As shown in Figure 6B, the increase of the percentage of T cells increased over 10 fold after the vaccination in the old mice.

Figure 6B

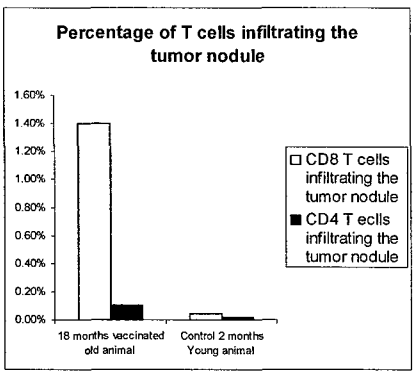
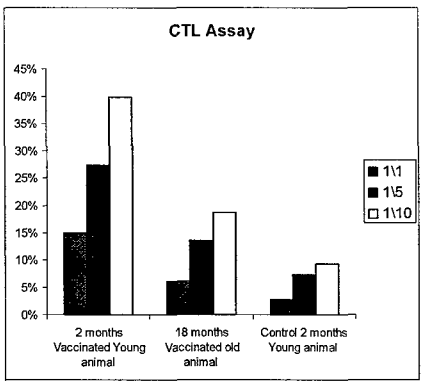


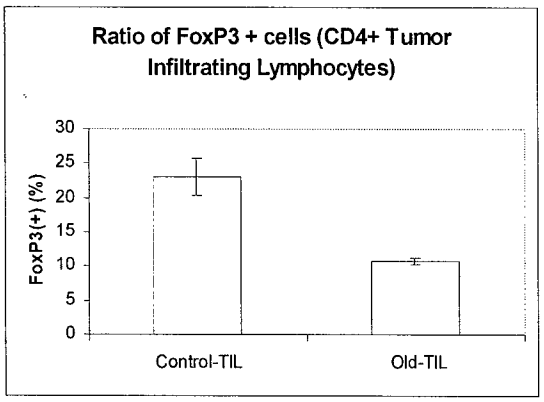
Figure 6C



We then tested the level of increase of antigen specific cytotoxic T cells induced by vaccination in 2 month and 18 month old mice. The results presented in Figure 6C show impressive increases in antigen specific CTLs following vaccine in the old as well as the young animals. Again, the level of the increase of the were less seen in the 19 month old mice was less than those seen in the 2 month old mice, but the absolute magnitude of the induction was impressive in the 18 month aged mice.

II.C.3.b. Effect of the Ad-sig-TAA/ecdCD40L Vector Vaccination on the Levels of CD4 FOXP3 Negative Regulatory T Cells in Tumor Tissue of Old Mice. Increases CD4 FOXP3 negative regulatory T cells in tumor tissue have been reported to limit the degree to which vaccines suppress the growth of tumor cells. Decreases in the level of FOXP3 negative regulatory CD4 T cells have been reported with vaccination. We therefore measured the level of FOXP3 CD4 T cells in the tumor tissue in old mice before and after Ad-sig-E7/ecdCD40L vaccination. As shown in Figure 7, the Ad-sig-E7/ecdCD40L vaccination decreased the percentage that the CD4 FOXP3 negative regulatory T cells constituted of the total CD4 cells in the tumor tissue in old mice by 2 fold.

Figure 7



II.C.3.c. Effect of the Ad-sig-TAA/ecdCD40L Vector on Tumor Growth in Old Mice. As shown below in Figure 8A, the vector induced suppression of E7 positive tumor growth in the 18 month old mice was almost equal to the level of suppression of the tumor growth in 2 month old mice. We then tested the effect of the protein boosts on the induction of the immune response induced by the Ad-sig-E7/ecdCD40L vector. The endpoint of these studies was in vivo suppression of the E7 tumor growth in C57Bl/6J mice, as measured by the percentage of mice which remained tumor free. As shown below in Figure 8B, the SC injection of the E7/ecdCD40L protein boost induced regressions of existing tumor and converted several tumor positive old (18 month) mice to tumor free mice. These data suggested that the E7/ecdCD40L protein boost could induce complete regressions in existing tumor which was progressive in 18 month old mice.

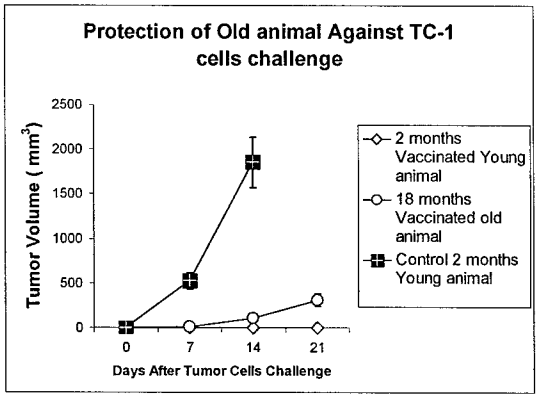


Figure 8A

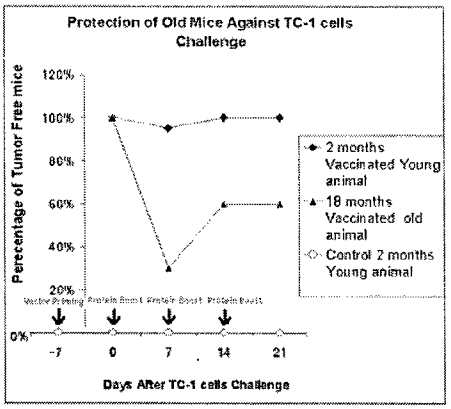


Figure 8B

III. Key Research Accomplishments

1. Demonstration that the vaccine works in older test animals
2. Demonstration that the vaccine induces an immune response against tumor vascular endothelial cells

IV. Reportable Outcomes

1. Demonstration that the vaccine works in older test animals
2. Demonstration that the vaccine induces an immune response against tumor vascular endothelial cells
3. Initiation of the toxicology/pharmacology/biodistribution for a clinical phase I trial of the vaccine.
4. Arrangements for the GMP manufacturing of the Ad-sig-TAA/ecdCD40L vector for the phase I trial of the vaccine.
5. Submission of provisional patent applications for the Ad-sig-TVECA/ecdCD40L vaccine strategy and the use of the Ad-sig-TAA/ecdCD40L for influenza vaccines as well as cancer vaccines in older people.
6. Publications of 2 papers and completion of 3 more manuscripts.

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V. Conclusions

1. The Ad-sig-TAA/ecdCD40L strategy works in tumor bearing older mice and therefore is suitable for clinical use in breast cancer.
2. The Ad-sig-TAA/ecdCD40L strategy is suitable for all vaccines (even for infectious diseases) in older people.
3. The Ad-sig-TAA/ecdCD40L strategy can induce an immune response against tumor vascular endothelial cell antigens (TVECA). The Ad-sig-TVECA/ecdCD40L and the Ad-sig-TAA/ecdCD40L vectors, when used together produce greater suppression of breast cancer growth than either vaccine alone.

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APPENDIX COVER PAGE

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Title: Intratumoral Injection of Chemotherapy Vector and Vector Activated Dendritic Cells Suppress Metastatic Breast Cancer.

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ABSTRACT

In the current study, we tested the effect of adding intratumoral (i.t.) vector (AdCDIRESE1A) directed chemotherapy on the immune response induced by i.t. injection of adenoviral vector activated dendritic cells (DCs) vaccine in a syngeneic mouse model. Our results show that the addition of i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector, which encodes the cytosine deaminase (CD) chemotherapy sensitization transcription unit, to the i.t. injection of DCs activated by the Ad-sig-CD40L vector, increases the killing of the CCL-51 syngeneic breast cancer cells in BalbC mice locally as compared to the administration of the DC vaccine or the AdCDIRESE1A chemotherapy sensitization vector alone. Moreover, the i.t. injection of Ad-sig-ecdCD40L vector infected DCs with the AdCDIRESE1A chemotherapy sensitization vector into subcutaneous CCL-51 breast cancer nodules suppressed the growth of uninjected metastatic tumor nodules in the lung. Finally, the combination of i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector to the i.t. administration of DCs infected with a rat Her-2-Neu (rH2N) expressing vector (Ad-sig-rH2N/ecdCD40L) led to the induction of rH2N specific anti-tumoral immunity in rH2N.Tg mice (which are anergic to the rH2N antigen) which suppressed the growth of established H2N positive NT2 breast cancer more efficiently than the vector targeted chemotherapy or the Ad-sig-rH2N/ecdCD40L infected DC vaccine alone. These results suggest that the simultaneous i.t. administration of tumor cell damaging agents with

vaccinations designed to TAA load and activate DCs may lead to a TAA specific immunity which can suppress existing deposits of breast cancer throughout the body.

INTRODUCTION

Many strategies have been proposed to overcome the anergy of host immune system against tumor associated antigens (TAA). If properly activated, dendritic cells (DC) may play a pivotal role in the development of anti-tumor immunity by presenting tumor antigens to and activating CD8+ cytotoxic T cells and CD4+ T helper cells (1-3). It has been proposed that the dying cancer cells might provide a danger signal that could alert the immune system to the presence of the cancer cells, not unlike that generated by bacterial or viral infections (4-10). Recent studies have also explored ways of using DC`s for the induction of an effective TAA specific immune response against cancer cells (11-16).

We decided to test if the combination of the adjuvant effect of dying tumor cells with the Ad-sig-TAA/ecdCD40L vaccine would increase the magnitude of the anti-tumor immune response induced by DCs. We had previously shown that the Ad-sig-TAA/ecdCD40L adenoviral vector induced a strong and durable TAA specific T-cell response against E7, Her-2-Neu (H2N) or human MUC-1 (hMUC1)-positive tumor cells (17-18).

In the present work, we transduced DCs with the Ad-sig-TAA/ecdCD40L vector vaccine and cancer cells of human or mouse origin with the AdCDIRESE1A vector. We had previously studied the efficacy of the AdCDIRESE1A in breast cancer cells (19-24).

Although the transfectional efficiency of adenoviral vectors in murine cells was not as high as in human cell lines, our work with mouse breast cancer cell lines showed that the AdCDIRESE1A adenoviral vector could destroy the mouse breast cancer cells. Secondly, we tested the in vivo efficacy of combining of the i.t. injection of the AdCDIRESE1A/5FC system with i.t. injection of adenoviral vector transduced DCs in syngeneic breast cancer models. Our results show that addition of i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector to the i.t. injection of Ad-sig-TAA/ecdCD40L transduced DCs increased the magnitude of a TAA specific anti-tumoral T cell response over that induced by either the AdCDIRESE1A/5-FC treatment or the administration of the Ad-sig-TAA/ecdCD40L vector infected DC vaccine alone. Importantly, the i.t. injection of the chemotherapy sensitization vector and the vector infected DC vaccine in subcutanteous (sc) tumor nodules induced a systemic immune response that suppressed the growth of uninjected metastatic pulmonary breast cancer nodules.

MATERIALS AND METHODS

Cell Lines and Mice.

The MCF-7 and CCL-51 human and mouse breast cancer cell lines, the HEK293 kidney cancer cell line, and the HTB-38 and CRL 2638 human and mouse colon cancer cell lines were purchased from American Type Culture Collection (ATCC). The OVCAR-5 human ovarian cancer cell line was obtained from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. The NT2 rat her-2-neu (rH2N) positive mouse breast cancer cell line was obtained from Dupont, Inc. Eight-week-old BalbC mice were purchased from Harlan. rH2N transgenic mice (rH2N.Tg mice) were obtained from Dupont, Inc. and then bred on site.

Construction of Recombinant Adenoviruses.

Wild type Adenovirus type 5 (Ad5WT) had been obtained from the American Type Culture Collection (ATCC). AdCDIRESE1A) was previously engineered in our laboratory (22-24), using the AdEasy vector system (25) and tittered as described previously (26).

Purification and Activation of Bone-Marrow Derived DCs.

Dendritic cells were induced to develop in vitro and purified from bone marrow cells as described previously (18), and activated with vectors at 50 MOI for 1 hour at 37°C.

Analysis of Infection Efficiency of Adenoviral Vectors in Mouse Cells.

The level of the expression of CAR, alpha V beta 3/5 integrin receptors , PDGFR-alpha and PDGFR-beta on the membrane of tumor cells were measured quantitavely by flow cytometric analysis as outlined previously (18, 22, 23). Transduction efficiency of Ad-sig-GFP vectors in cell lines and DCs was determined following a 48 hour in vitro exposure by flow cytometry (18, 22, 23).

Western Blot Analysis of E1A Proteins.

Forty-eight hours after vector infection, tumor cells were heated at 100°C for 5 minutes and then 20 ul of samples were loaded on to the pre-prepared gel. The Western was developed as ourlined previously (17-18, 22-23).

Virus Yield Assay.

Cells were infected with AdCDIRESE1A at MOI 1in 1 ml of growth medium supplemented with 1%FBS. Following an 1 hour incubation at 37°C, cells were washed. After 5 days in 2.5% FBS supplemented mdium, the supernanatant medium from the vector infected cells was used for viral titration. The number of infectious adenoviral particles was determined by both limiting dilution assay of plaque formation in HEK293 cells and plaque assay as described previously (23).

Analysis of the Expression of the CD gene in the Ad-sig-CD and AdCDIRESE1A Vectors.

The expression of the *CD* gene in the bicistronic transcription unit of the AdCDIRESE1A vector was measured as described previously (20-23) by extracting RNA from vector infected cells infected with the vector and used to generate cDNA with the following primers: Xho I (ccgctcgagaggctaatgtcgaat) and Xba I (gctctagattaccgtttgtaatcgat) using ''Superscript-II reverse transcriptase'' enzyme at 25°C for 10 minutes. After treatment with Rnase H at 37°C for 20 minutes, the first strand cDNA was amplified by using Ready to go PCR-beaded tubes (Amersham Pharmacia Biotech) for 30 cycles. Each cycle included a denaturation period of 30 seconds at 94°C and an annealing period of 60 seconds at 54°C. The predicted molecular weight of the *CD* fragment generated by this PCR reaction is 1.2 Kb.

Functional Analysis of the CD Gene in the Adenoviral Vector Backbone.

Cells were exposed to the AdCDIRESE1A vector at the following MOI: 0, 1, 10, 40, and 80. After 24 hours, sufficient 5-Fluorouracil (5FC) from Sigma Chemical Co. was added to make the cultures 500 micromolar. After 3 days, the MTT assay (MTT cell proliferation assay, ATCC) was performed according to the manufacturer`s instructions.

Tests for the Cellular Immune Response in the Test Mice.

T cells were isolated as previously outlined (17-18). ELISPOT, cytotoxicity, and cytokine release assays were carried out as previously reported (17-18).

Animal Studies.

Mouse Model #1: **Effect of AdCDIRESE1A Vector on Ad-sig-CD40L Infected DC Induced Suppression of Injected CCL-51 Breast Cancer SC Tumor Nodules in BalbC Mice.** $1x10^6$ CCL-51 cells were injected sc into BalbC mice. When the tumor nodule reached the 100 mm^3 range, 10^8 pfu of the AdCDIRESE1A vector or the same volume of PBS were injected intratumorally (i.t.) into 6 mice for each of the treatment groups. The mice were given a 10 day course of intraperitoneal (ip) therapy (PBS or 5FC at 500mg/kg) to the groups defined below in Table 1. Five hundred thousand vector infected DCs were injected into the tumor nodules 3 days after the i.t. injection of the

PBS or AdCDIRESE1A vector. The treatment groups are shown in Table 1. The goal of this experiment is to test the effect of adding it injection of the AdCDIRESE1A vector to i.t. injection of Ad-sig-CD40L vector infected DC induced suppression of the injected sc tumor nodules.

Mouse Model #2: **Effect of the Addition of the I.T. Injection of AdCDIRESE1A Vector to the I.T. Injection of Ad-sig-rH2N/ecdCD40L Infected DC Induced Suppression of Injected NTS Breast Cancer SC Nodules in rH2N.Tg Mice.** We tested the same strategy as outlined in Mouse Model #1 but with the i.t. injection of DCs infected with the Ad-sig-rH2N/ecdCD40L vector into rH2N positive NT2 breast cancer sc tumor nodules in 16 week old rH2N.Tg mice. The goal is to test the effect of adding i.t. injection of the AdCDIRESE1A vector on the magnitude of the rH2N specific immune suppression of sc tumor rH2N positive NT2 sc tumor nodules induced by i.t. injection of Ad-sig-rH2N/ecdCD40L infected DCs.

Mouse Model #3: **Effect of the Addition of I.T. Injection of the AdCDIRESE1A Vector on Suppression of Distant Uninjected CCL-51 Metastatic Pulmonary Nodules by the I.T. Injection of the Ad-sig-CD40L Vector Infected DCs Into SC Tumor Nodules.** In order to test the remote systemic effect of the combination of the i.t. injection into sc CCL-51 tumor nodules of the AdCDIRESE1A with and Ad-sig-ecdCD40L infected DCs BalbC mouse model, 3 groups of BalbC mice similar to the groups #1, #3 and #5 of mouse model #1, which were designated as group #3-1, #3-2 and #3-3, were treated in a manner similar to that outlined for groups #1, #3, and #5 of

mouse model #1 (see Table 1). Balb C mice were injected subcutaneously with 500,000 CCL-51 breast cancer cells. Two weeks after DC injection, $1x10^5$ CCL-51 breast cancer cells were injected through tail veins of the mice to generate pulmonary nodules. Following an additional four weeks, the mice were sacrificed and the number of tumor nodules in the lungs was counted.

Statistical Analysis.

Results of the in vitro cytotoxicity tests were evaluated by the Student's T test. SPSS-10.0. One-way ANOVA (with LSD post-hoc comparisons) and Mann-Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis.

RESULTS

Tests for Infectivity of Human and Mouse Cell Lines by Adenoviral Vectors.

A. Level of Expression of CAR, the Alpha V Beta 3/5 Integrin Receptor, the PDGFR-α, and the PDGFR-beta in Human and Mouse Cancer Cells.

To evaluate the potential for adenoviral binding and infection of human and mouse tumor cells, we measured the level of the CAR, and the alpha V beta 3/5 integrin receptors which mediate the cellular binding and uptake of adenoviral vectors on tumor cells. We also studied two additional receptors: PDGFR-α and PDGFR-β, which have been proposed to play a role in transduction of adenoviral particles into cells. The percentage of cells which were positive for the alpha V beta 3/5 integrin receptors, the CAR, the PDGFR-α and the PDGFR-β receptors was measured by flow cytometry after staining the cells with antibodies specific to these receptors followed by exposure to secondary FITC-conjugated IgG antibodies.

As shown in Table 2, all of the human tumor cell lines expressed the receptors for CAR and alpha V beta 3 integrin receptor, both of which are known to be important for the adenoviral infections in mouse and human cell lines. The level of CAR and alpha V beta 3/5 integrin receptors was higher in the OVCAR-5 and HTB-38 human cell lines than in the other human and mouse cell lines tested (see Table 2). The level of the PDGFR-α and the PDGFR-β receptors was higher in the OVCAR-5 cell line than in the

other human and mouse cell lines tested. On the basis of these data, we predict that both the human and the mouse cell lines should be infectable with the adenoviral vectors under study.

B. Analysis of GFP Expression.

As shown in Figure 1B, the Ad-sig-GFP vector was capable of transducing both mouse DCs and tumor cell lines. As shown in Figure 1C, however, the transduction efficiency in mouse cells was not as high as in human cell lines. The number of GFP expressing rH2N positive NT2 mouse breast cancer cells was found to be as high as in human cell lines if the MOI used in the experiments was increased.

C. Western Blot Analysis of E1A Protein Expressed by the Tumor Cells Infected with E1A Encoding Vectors.

Cell lines were seeded at a density of 100,000 cells/well in 6-well plates. Twenty-four hours later, these cells were exposed to the following vectors, Ad-sig-CD, Ad-sig-E1A, Ad-sig-E1A, AdCDIRESE1A and the AdWT virus. After 2 days of incubation, the tumor cells were harvested, and the lysates of these cells were studied for the level of E1A protein expression. The bands produced by Western blotting from CCL-51 cells are shown in Figure 1D. All the cell lines used in the current study yielded similar results. The bands specific for E1A protein, which were obtained from the Ad-sig-E1A vector infected cells show the expected molecular weight range for E1A peptides, which is around 30 KDa. In contrast, no protein bands are visible from the uninfected control cells

or from the cells infected with non-replicating vectors. This data shows that the adenoviral vectors (with CMV promoters) had the capability of expressing E1A protein in the tumor cells.

D. Virus Yield Assay.

In order to compare the production of virus progeny from mouse tumor cells with that from human cell lines, we carried out a virus yield assay. The viral progeny produced at the end of a 5-day infection period were tittered. Mouse tumor cell lines were found to produce significantly lower levels of infectious viral particles than seen with human tumor cells (see Figure 1E). This result shows that replication competent adenoviral vectors could infect the mouse tumor cell lines, but they cannot replicate as efficiently as in human cell lines.

E. Analysis of the Expression of the CD gene in the Ad-sig-CD40L and AdCDIRESE1A Vectors.

The following cell lines were seeded at a density of 200,000 cells/well in six-well plates: MCF-7, OVCAR-5, HTB-38, CCL-51, NT2, and CRL 2638. Twenty-four hours later, these cells were then incubated with the following vectors for a 16 hour period of incubation: AdCDIRESE1A, Ad-sig-E1A and Ad-sig-CD. Then, the cells were trypsinized and washed with PBS. Total RNA was then isolated from these cells, and cDNA was then generated using the primers specific for the CD coding transcripts.

Portions of the *CD* gene were synthesized and amplified from the mRNA of the gene by RT-PCR. The bands produced by PCR from the cDNA of CCL-51 cells are shown in Figure 1F. All the cell lines used in the current study yielded similar results.The analysis of the bands produced by the amplification of the RNA from the Ad-sig-CD vector infected cells show the expected molecular weight for *CD* (1.2 Kb). In contrast, the expected *CD* fragments were not seen in the RNA from the cells infected by the control vectors. These data show that the AdCDIRESE1A and Ad-sig-CD vectors were expressing *CD* coding mRNA sequences.

F. In Vitro Functional Analysis of the CD Gene in the Adenoviral Vector Infected Cells.

We then analyzed the cytotoxicity generated in vitro by vectors at different MOI in tumor cell lines derived from human carcinomas of the breast, ovary and mouse breast cancer. In this study, in vitro cytotoxicity tests were carried out with the replication-competent AdCDIRESE1A vector. As shown in Figure 1G, the maximum predicted cytotoxic effect of the replication-competent AdCDIRESE1A vector without 5FC treatment (dotted lines in Figure 1G) at the maximum doses was around 50% in mouse as well as in the human tumor cells so studied (see Figure 1G). As shown by the data presented in Figure 1G by the solid bold line, the addition of 5FC caused a significant increase in cytotoxicity in all human and mouse tumor cell lines so studied ($p<0.01$). The maximum predicted cytotoxicity after addition of 5FC to the AdCDIRESE1A vector infected cells was over 90% in MCF-7 (human) and OVCAR-5 (human). It was over 80% in the NT2 mouse breast cancer cell line and over 70% in the CCL-51 mouse breast

cancer cell line. The two therapeutic transcription units (*CD* and *E1A*) together appear to be additive in the effect of vectors on both human and mouse tumor cells (see Figure 1G).

Tests for In Vivo Induction of the Cellular Immune Response.

A. Cytokine Release from Splenic T-cells of Vaccinated Mice.

In order to test the efficacy of the various strategies for inducing an immune response, we measured the cytokine release from the activated splenic T cells isolated mice in the different test groups listed in Table I. As shown in Figure 2A, the T-cells from mice i.t. injected with the combination of the vector infected DCs and the AdCDIRESE1A chemotherapy sensitization vector, released significantly more IFN-gamma than did groups injected with pre-infected DCs alone or the AdCDIRESE1A alone. However, there was no significant difference between the treatment groups in terms of GM-CSF released from activated T-cells ($p>0.05$) as shown in Figure 2A.

B. The Frequency of IFN gamma and IL-4 Secreting T Cells from the Spleens of Vaccinated Mice.

The frequency of IFN-gamma and IL-4 secreting splenic T cells of the experimental treatment groups of mice was assessed by ELISPOT assay. As shown in Figure 2B, mice injected with a combination of vector infected DCs and the AdCDIRESE1A chemotherapy vector (group #5) had significantly more IFN-gamma

(80±14) and IL-4 (35±12) secreting T-cells when compared to mice i.t. injected with vector infected DCs alone or the chemotherapy vector alone ($p<0.001$).

The lower number of the spots for IL-4 secreting cells, when compared to IFN-gamma spots, suggest that the DC vaccination plus AdCDIRESE1A vector treatment stimulates a Th1 rather than a Th2 immune response.

C. Cell Mediated Cytotoxicity Assay of Splenic T Cells From the Vaccinated Mice.

Cell mediated cytotoxicity was assayed by an antibody to the apoptosis associated caspase-3 by flow cytometry. Seven days after the injection of DCs, the spleens of the mice were removed and CD8 (+) cells were isolated. The CD8 (+) cells from mice injected i.t. with the AdCDIRESE1A chemotherapy vector plus i.t. DCs showed significantly higher cytotoxicity against tumor cells than CD8 (+) cells from mice injected with the AdCDIRESE1A vector alone or the vector infected DCs alone (see Figure 2C).

Effect of I.T. Injection of the AdCDIRESE1A Chemotherapy Sensitization Vector on the Immune Response Induced by the I.T. Injection of Vector Infected DCs.

A. Effect of I.T. of AdCDIRESE1A Chemotherapy Sensitization Vector on the Immune Suppression Induced by IT Injection of Ad-sig-CD40L Vector Infected DCs of SC Nodules of CCL-52 Breast Cancer Cells in BalbC Test Mice-Mouse Model #1.

The effect of i.t. injection of the AdCDIRESE1A/5FC system in combination with the i.t. injection of DCs infected with the Ad-sig-CD40L vector was tested in BalbC mice bearing sc tumor nodules derived from the CCL-51 mouse breast cancer cell line. On the seventh day of following injection of CCL-51 cells, the tumor volumes were measured and the mice were randomly divided into 5 groups:

1) mice injected i.t. with PBS served as control group (Group #1-1;

2) mice injected i.t. with PBS then followed in 3 days by i.t. injection of DCs infected ex vivo with the Ad-sig-GFP vector (Group #1-2);

3) mice injected i.t. with PBS then followed in 3 days by i.t. injection of DCs infected ex vivo with the Ad-sig-CD40L (Group #1-3);

4) mice injected i.t. with AdCDIRESE1A chemotherapy vector followed by ip 5FC (Group #1-4);

5) mice injected i.t. with the AdCDIRESE1A chemotherapy vector followed by ip 5FC followed in 3 days after the chemotherapy vector injection by i.t. injection of DCs infected ex vivo with the Ad-sig-CD40L vector (Group #1-5).

The mice in all groups were followed until death or sacrifice because of large tumor volume. In BalbC mice treated with combined i.t. injection of vector infected DCs and the AdCDIRESE1A/5FC chemotherapy vector (Group #1-5), all the tumor nodules of have disappeared after the second week of the treatment. There were only a small number of complete remissions in the other test groups (Groups #1-2, #1-3 and #1-4). The vector targeted chemotherapy group (those receiving the AdCDIRESE1A plus ip 5-

FC) without vector infected DC i.t. injection had the second best tumor response pattern. I.T. injection of vector infected DCs alone caused a lower frequency of partial and complete tumor responses in Groups #1-2 and #1-3.

We then studied the survival of the teated BalbC mice following subcutaneous injection of the NT2 breast cancer cells and vaccination. As shown in Figures 3A and 3B, there was no significant difference of survival in the groups injected by with DCs infected with the Ad-sig-GFP or the Ad-sig-CD40L vectors, and groups injected by the DCs not infected with vectors (See Groups #2 and #3 in Figure 3A and 3B). The combination of the i.t. injection of the AdCDIRESE1A chemotherapy vector with i.t. injection of DCs infected with the Ad-sig-CD40L vector produced the best survival pattern of any of the test groups (see Figure 3B).

B. Effect of the Addition of the I.T. Injection of AdCDIRESE1A Vector to the I.T. Injection of Ad-sig-rH2N/ecdCD40L Infected DC Induced Suppression of Injected rH2N Positive NT2 Breast Cancer SC Nodules in rH2N.Tg Mice -Mouse Model #2.

We studied the combination of i.t. injected AdCDIRESE1A chemotherapy vector plus DCs infected with the Ad-sig-rH2N/ecdCD40L adenoviral vector in rH2N.Tg transgenic mice bearing sc tumor nodules derived from the rH2N positive NT2 mouse breast cancer cells. The experimental design similar to that used in Mouse Model #1. However, the DCs were infected ex vivo with the Ad-sig-rH2N/ecdCD40L vector in

Group #2-3 and Group #2-5. The rH2N.Tg mice injected i.t. in sc tumor nodules derived from the NT2 breast cancer cells with the AdCDIRESE1A chemotherapy vector plus Ad-sig-rH2N/ecdCD40L vector infected DCs (Group #2-5) suppressed the tumor growth completely (see Figure 3C). Again, the combination of i.t. injection of the AdCDIRESE1A chemotherapy vector combined with the i.t. injection of the Ad-sig-rH2N/ecdCD40L vector infected DCs produced the best survival pattern in this model (see Figure 3D).

C. Mouse Model #3: Effect of the Addition of I.T. Injection of the AdCDIRESE1A Vector on Suppression of Distant Uninjected CCL-51 Metastatic Pulmonary Nodules by the I.T. Injection of the Ad-sig-CD40L Vector Infected DCs Into SC Tumor Nodules. - *Mouse Model #3:*

To test whether the immunity elicited by the combined i.t. local injection of a chemotherapy sensitization vector and DCs infected with the Ad-sig-CD40L vaccine into sc CCL-51 breast cancer nodules could could induce a systemic immune response that would suppress uninjected distant pulmonary tumor nodules, we established 3 groups of mice: Groups #3-1, #3-2 and #3-3, which were similar to the Groups #1-1, #1-3 and #1-5 of the mouse model #1 (see Table 1). Two weeks after i.t. injection of DCs, all of the groups were rechallenged by 1 x 10^5 CCL-51 cells through tail vein injection with the intent of generating pulmonary nodules in the lungs of the BalbC test mice. Four weeks following the iv injection of the CCL-51 cells, the mice were sacrificed, and the number of pulmonary nodules of CCL-51 breast cancer was counted.

All the mice in the Groups #3-1 (no treatment) and #3-2 (DCs activated with Ad-sig-CD40L) had tumor nodules in the lung. However, there were fewer tumor deposits in the lungs of the mice in Group #3-2 which were injected i.t.with DCs infected with the Ad-sig-CD40L vector than in Group #3-1 in which no vaccination was given. The average counts for the tumor nodules in the lungs of the control group (Group #3-1-no vaccination) was over 50. However, there were no tumor depsoits seen in any of the mice of the Group #3-3 treated with local i.t. injection into sc tumor nodules of the AdCDIRESE1A chemotherapy vector plus i.t. injection into sc tumor nodules of the DCs infected by the Ad-sig-CD40L vector. This result shows that the immunity induced by the local treatment of the sc CCL-51 tumor nodules with AdCDIRESE1A chemotherapy vector plus DCs infected ex-vivo with the AdCD40L vector induced a systemic immune response which could prevent engraftment of uninjected distant pulmonary tumor nodules derived from the CCL-51 breast cancer cells.

DISCUSSION

Animals previously treated with the AdCDIRESE1A/5-FC system or tumor cells transfected with the *CD* gene and then treated with 5FC, have been reported to be resistant to subsequent challenge with wild type tumor cells (not containing the *CD* gene). These results have raised the possibility that a systemic immune response can be developed against the parental tumor cells following treatment with *CD* modified tumor cells (27). Study of the i.t. injection of DCs infected with an adenoviral vector encoding the IL-12 gene, both in animal model and in the clinic in human subjects has shown the induction of a potent immune response and regressions in pancreatic cancer (28-30).

Previous reports have shown the i.t. injection of sc tumors with adenoviral vectors carrying the *CD* gene/5FC system can suppress the growth of the injected subcutaneous tumor cell lines in vivo models (21-23, 31-33). The vector infection efficiency of adenoviral vectors in mouse cell lines is not as high as in human cells. In spite of this disadvantage, we showed that the adenoviral vectors can infect mouse cells sufficiently to kill the established tumor deposits (see Figure 3). It has been known that the apoptosis seen in tumor cells induced by conventional chemotherapy was associated often with the induction of a tumor specific immune response (37).

Although the exact mechanism of the effect of dying tumor cells on the induction of an anti-tumor immune response is not completely understood, DCs are thought to play

an important role (33). We have therefore tested whether it is possible to increase the level of the anti-tumor immune response induced by i.t. injection of adenoviral infected DCs by the i.t. injection of AdCDIRESE1A chemotherapy vector/5-FC system. One potential problem for the design of these experiments was the low infection efficiency of adenoviral vectors in mouse cells. This lower transduction efficiency of DCs has prompted researchers to try to induce DCs in ways other than use of adenoviral vectors.

We observed a dramatic increase in GFP transgene expression in Ad-sig-GFP exposed mouse DCs by increasing the number of infectious particles (see Figures 1B-1C). This shows that mouse DCs could easily be infected and activated ex-vivo by using adenoviral vectors. In vivo experiments in BalbC mice (mouse model #1) showed that the CCL-51 breast cancer bearing mice treated i.t. with the AdCDIRESE1A/5-FC system plus Ad-sig-CD40L vector infected DCs induced a substantial tumor specific T-cell response which was greater than that induced by the chemotherapy vector or the vector infected DCs alone (see Figure 2). This result indicates that tumor cell killing augments the immune response induced by a DC vaccine. In addition, the combination of i.t. injection of vector infected DCs and AdCDIRESE1A chemotherapy vector could produce an antigen non-specific immune response induced against tumor cells resulting in suppression of tumor cell growth and an extension of survival of test mice (see Figures 3A and 3B).

Similar experiments with an antigen specific vaccine (DCs infected with the Ad-sig-rH2N/ecdCD40L vector) also showed that the addition of the i.t. administration of the

AdCDIRESE1A chemotherapy sensitization vector to the i.t. injection of the Ad-sig-rH2N/ecdCD40L vector infected DCs increase the tumor response and the survival of the test mice (see Figures 3C and 3D) over that achievable with either the AdCDIRESE1A vector or the Ad-sig-rH2N/ecdCD40L vector infected DCs alone.

The metastatic nature of cancer requires that the impact of any treatment be distributed throughout the body. In a recent study, we have shown that local i.t. injection of chemotherapy sensitization vectors in a xenograft model of colon cancer resulted in the complete eradication of the injected tumor nodules when the AdCDIRESE1A/5-FC treatment system was combined with CPT-11 (23).

In order to translate these strategies into a vector treatment which could be administered locally, but would generated an immune response that would suppress uninjected distant metastatic disease, we studied the effect of adding the i.t. injection of sc tumor nodules with the AdCDIRESE1A chemotherapy sensitization vector to the i.t. injection into sc tumor nodules with the Ad-sig-CD40L vector infected DCs. The data presented in Figure 3E show that this combined local vector mediated chemotherapy and vaccine, induced a systemic immune response that was capable of suppressing uninjected distant pulmonary breast cancer nodules, 4 weeks following injection.

Developing methods for the targeting of vectors to tumor cells and their vasculature will be important in the effort to increase the efficacy of in vivo treatment with the gene therapy vectors or the combination of vector therapy with chemotherapy

when the vector is administered systemically (34-36). Recently, much effort has been devoted to improving the transfection efficiency of the gene therapy vectors for tumor cells. Our laboratory as well as others is currently focused on modifying the adenoviral vectors in ways that would increase the specificity and efficiency of delivery of these vectors to the target cells. In this report, we have focused on a local vector injection to generate a systemic response for the control of metastatic disease. The results outlined in this report are encouraging in that currently available adenoviral vectors can be used in animal models to produce substantial tumor responses of uninjected systemic disease.

Our results suggest that the use of i.t. administration of ex-vivo activated DCs in combination with the local use of a prodrug activating gene therapy vectors could at this time be used to treat metastatic tumors more efficiently than is possible with the current treatment modalities in patients whose disease is resistant to chemotherapy alone.

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Table 1. The Treatment Groups of the Mouse Models.

Groups Cell Line	Treatments Route
BalbC Model#1 CCL-51 • Group #1-1 • Group #1-2 • Group #1-3 • Group #1-4 • Group #1-5	SC • Control (PBS) • DC (activated with Ad-sig-GFP) • DC(activated with Ad-sig-CD40L) • AdCDIRESE1A+5FC only • AdCDIRESE1A+5FC+ DC (activated with the Ad-sig-CD40L vector)
rH2N.Tg Model#2 NT2 • Group #2-1 • Group #2-2 • Group #2-3 • Group #2-4 • Group #2-5	SC • Control (PBS) • DC (activated with Ad-sig-GFP) • DC (activated by Ad-sig-rH2N/ecdCD40L) • AdCDIRESE1A+5FC only • AdCDIRESE1A+5FC+ DC (activated With the Ad-sig-rH2N/ecdCD40L vector)
BalbC Model#3 CCL-51 • Group #3-1 • Group #3-2 • Group #3-3	IV • Control (PBS) • DC(activated with Ad-sig-CD40L) • AdCDIRESE1A+5FC+ DC (activated With the Ad-sig-CD40L vector)

Table 2. The Percentage of Tumor Cells Positive for the CAR, Alpha V Beta 3/5 Integrin Receptors, the PDGFR-α and the PDGFR-β Receptors as Measured by FACS Analysis.

Species	Cell lines	CAR (%)	αv (%)	PDGFR-α (%)	PDGFR-β(%)
Mouse	CCL-51	9.5±1.7	6.7±1.4	10.1±1.1	8.6±1.9
Mouse	NT-2	18.6±7.0	15.8±2.5	18.9±1.9	14.8±2.7
Mouse	CRL-2638	5.9±1.5	3.1±1.1	5.4±1.7	6.4±1.9
Human	MCF-7	13.2±3.9	19.6±2.9	12.7±1.4	11.6±1.6
Human	OVCAR-5	52.3±9.5	78.4±3.8	51.9±2.4	51.2±7.1
Human	HTB-38	50.5±15.3	80.5±5.7	7.8±1.2	13.7±2.15

FIGURE LEGENDS

Figure 1. Human adenoviral vectors can infect mouse cells.

A) Vectors used in the study.

B) Ad-sig-GFP transduces both mouse and human cell lines. B1: human tumor cell lines; B2: mouse cells.

C) Inverted fluorescent microscope images of *GFP* transgene expression in human and mouse cell lines. The mouse tumor cell lines expressed GFP but at a lower level than human cell lines this means that an adenoviral vector carrying a therapeutic transcriptional unit could efficiently transduce and express therapeutic genes in mouse cells.

D) Western blotting of E1A polypeptides produced in vector infected CCL-51 cells. The E1A region encodes a series of related peptides (35 – 46kDa).

E) Viral yield test results. The human tumor cell lines produced almost 1000 times more viral particle of the AdCMVCDIRESE1A vector than the mouse tumor cells.

F) Expression of *CD* gene in CCL-51 cells. (Lane 1: 1kb Molecular Weight Marker; Lane 2: AdCDIRESE1A; Lane 3: Ad-sig-E1A; Lane 4: Ad-sig-CD; Lane 5: Control cells; Lane 6: Control RNA a without reverse transcriptase).

G) Results of in-vitro cytotoxicity tests. The maximum cytotoxicity level of the vector used at the given MOI in mouse tumor cell lines was 50% when the AdCDIRESE1A vector was used without 5FC (dotted line), and was around 60% for the human cell lines. The addition of 5FC significantly increased the cytotoxicity of the vector in both human and mouse tumor cells (see solid bold line).

Figure 2. Induction of specific immunity by the i.t. injected DCs.

A) Cytokine release from the activated splenic T-cells of BalbC mice. The T-cells from the DC plus gene therapy with a replication competent vector carrying CD transcription unit released significantly more IFN-gamma than the other groups (**Group #1:** Control (PBS); **Group #2:** DC infected with Ad-sig-GFP vector); **Group #3:** DC infected with Ad-sig-CD40L vector ;**Group #4:** AdCDIRESE1A+5FC only; **Group #5:** AdCDIRESE1A+5FC + DC infected with the Ad-sig-CD40L vector

B) Results of ELISPOT analysis of mice. The mice injected with both DC and AdCDIRESE1A chemotherapy vector (Group #5) had significantly more IFN-gamma (80±14) and IL-4 (35±12) secreting T-cells than other groups.

C) Cell-mediated cytotoxicity of T-cells from the mice. T-cells from Group 5 mice showed significantly higher cell-mediated cytotoxicity than seen with T cells from other groups.

Figure 3. In vivo Efficacy of the combination of i.t. injection of AdCDIRESE1A +5-FC system and DCs.

A) **Effect of vaccine on tumor cell growth in BalbC mice injected sc with CCL-51 cells-mouse model #1.**

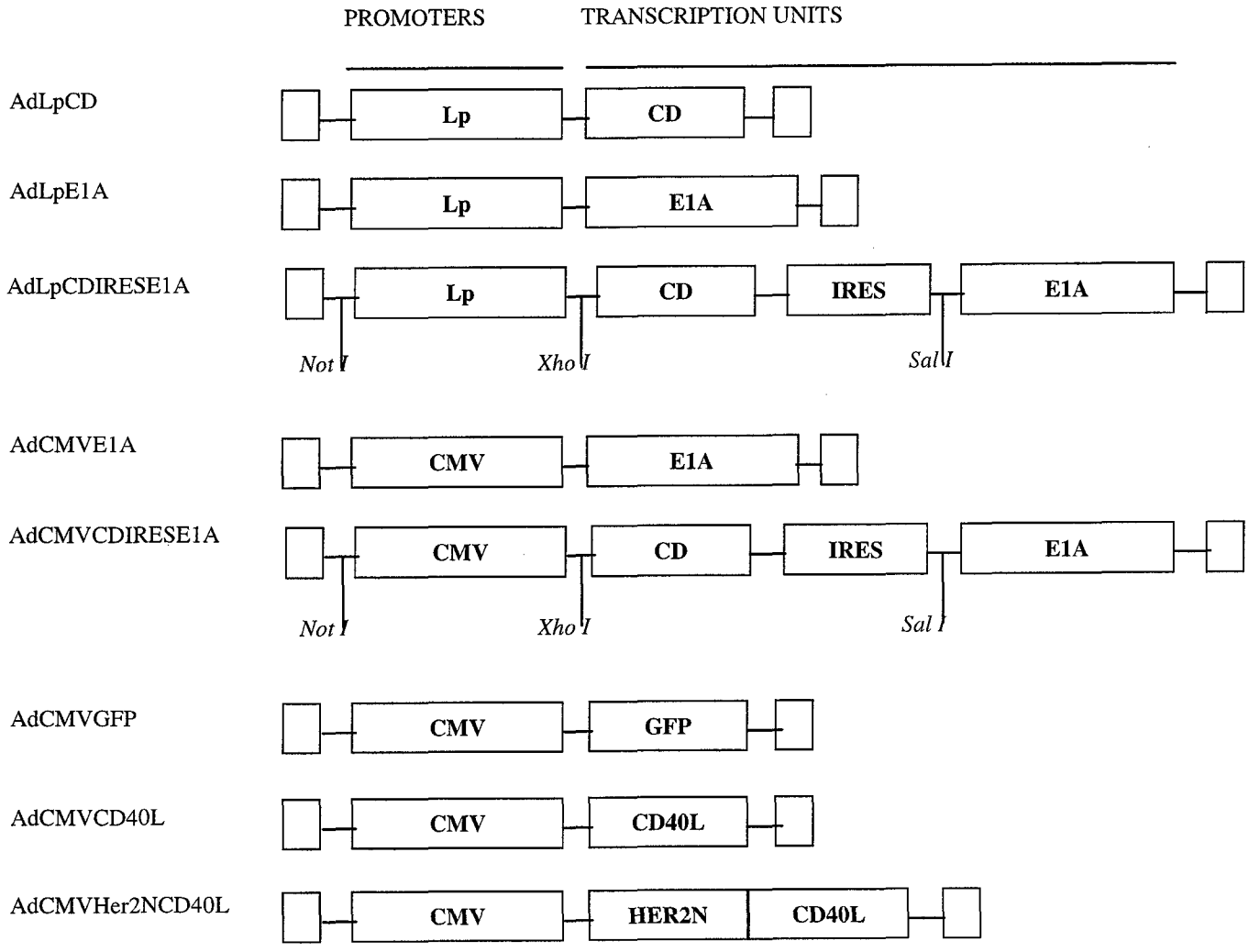
B) **Effect of vaccine on survival of BalbC mice injected sc with CCL-51 cells-mouse model #1.** Vector targeted chemotherapy plus DCs injected group had significantly longer survival than the other groups.

C) **Effect of vaccine on tumor cell growth in rH2N.Tg mice injected sc with NT2 cells-mouse model #2.**

D) Effect of vaccine on survival of rH2N.Tg mice injected sc with NT2 cells-mouse model #2. AdCDIRESE1A vector targeted chemotherapy plus Ad-sig-rH2N/ecdCD40L vector infected DCs injected group had significantly longer survival than the other groups. .

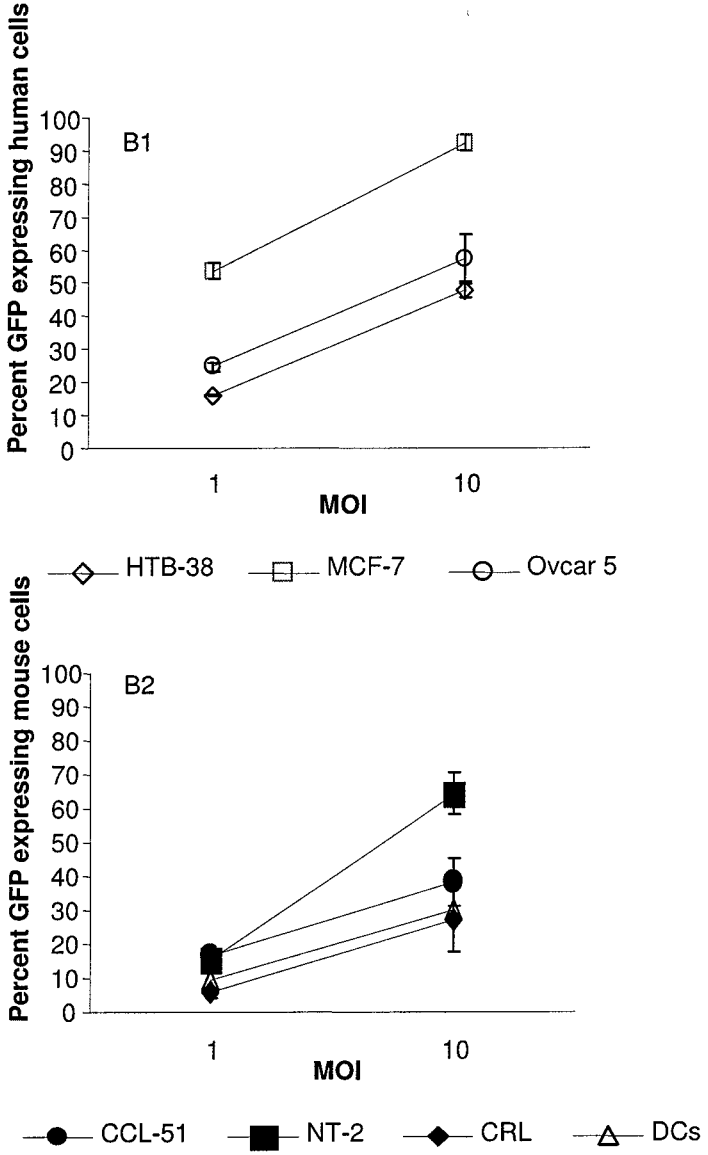
E) Effect of adding i.t. AdCDIRESE1A chemotherapy vector to Ad-sig-CD40L vector infected DCs administered to sc tumor nodules on the number of CCL-51 tumor nodules in the lung of the BalbC mice-mouse model #3.

Fig 1A Vector Maps



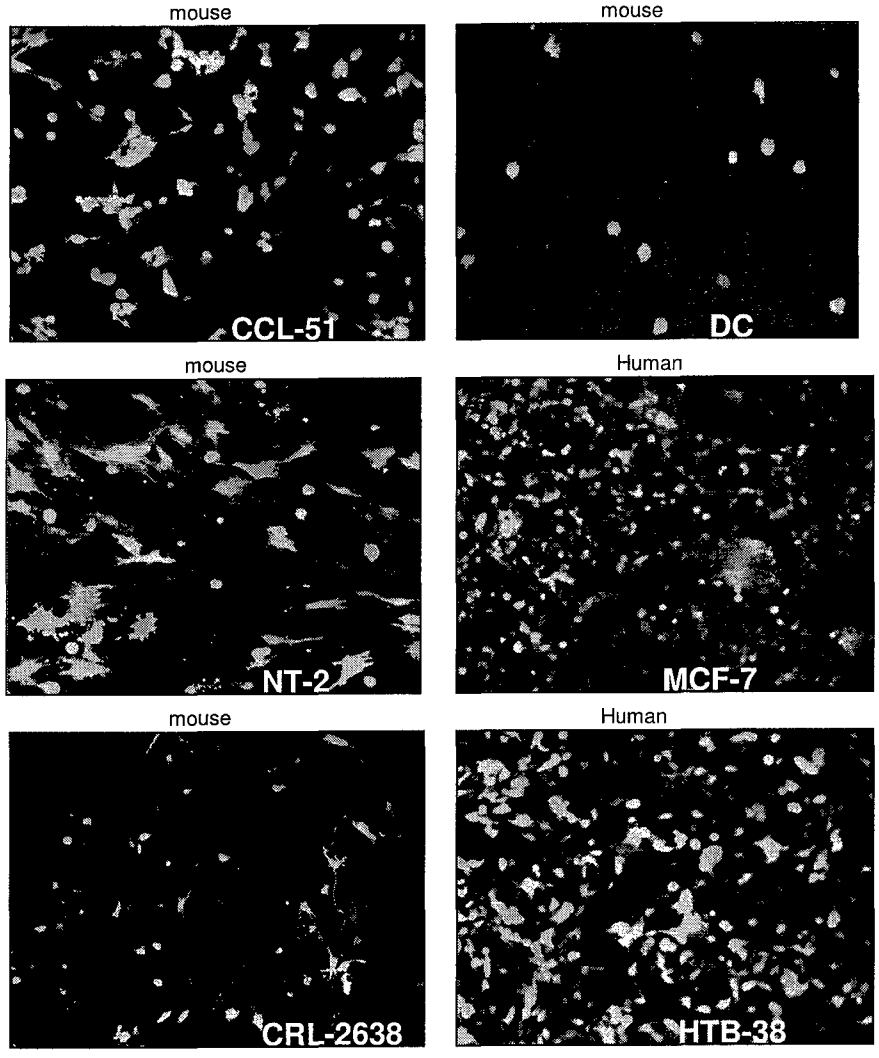
- Figure 1-

Fig 1B. Percentage of GFP positive cells in Ad-Sig-GFP Exposed Cells



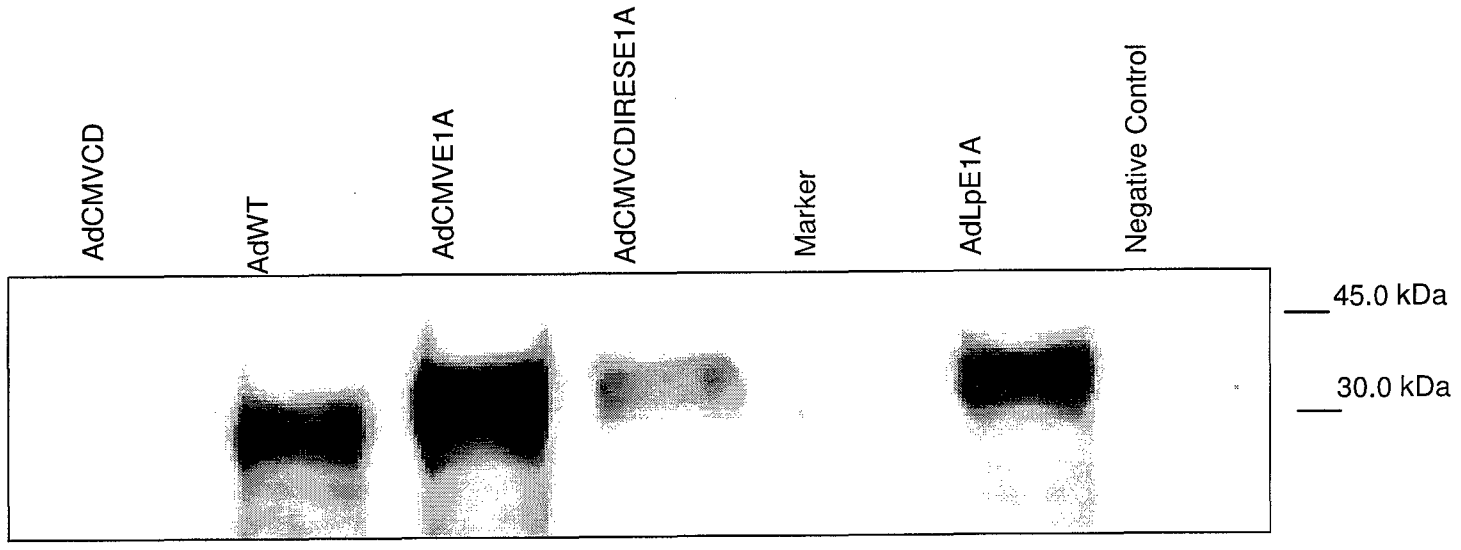
- Figure 1-

Fig 1C Flourescent Microscopy of Ad-Sig-GFP Vector Exposed Cells



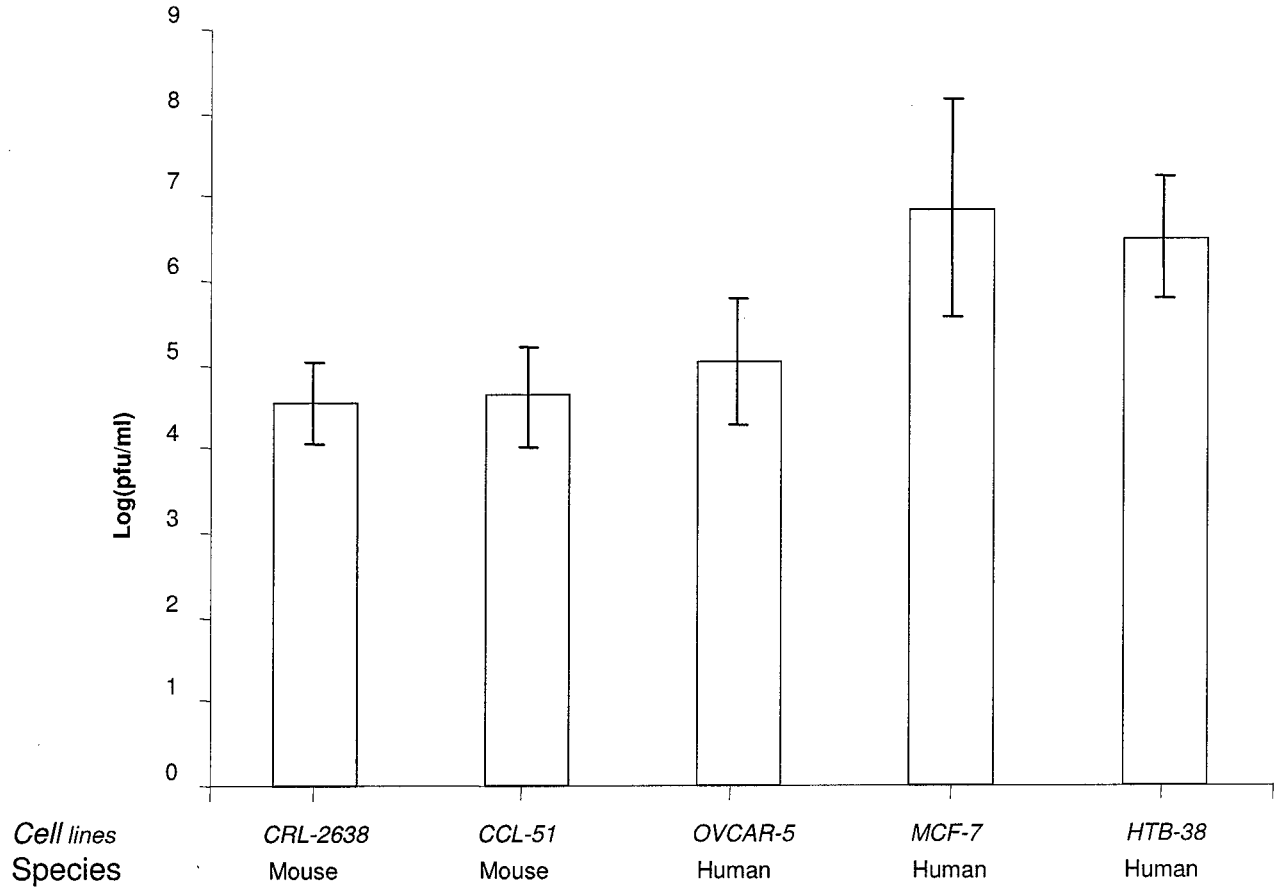
- Figure 1-

Fig 1D. Western Blotting Analysis of E1A Peptides



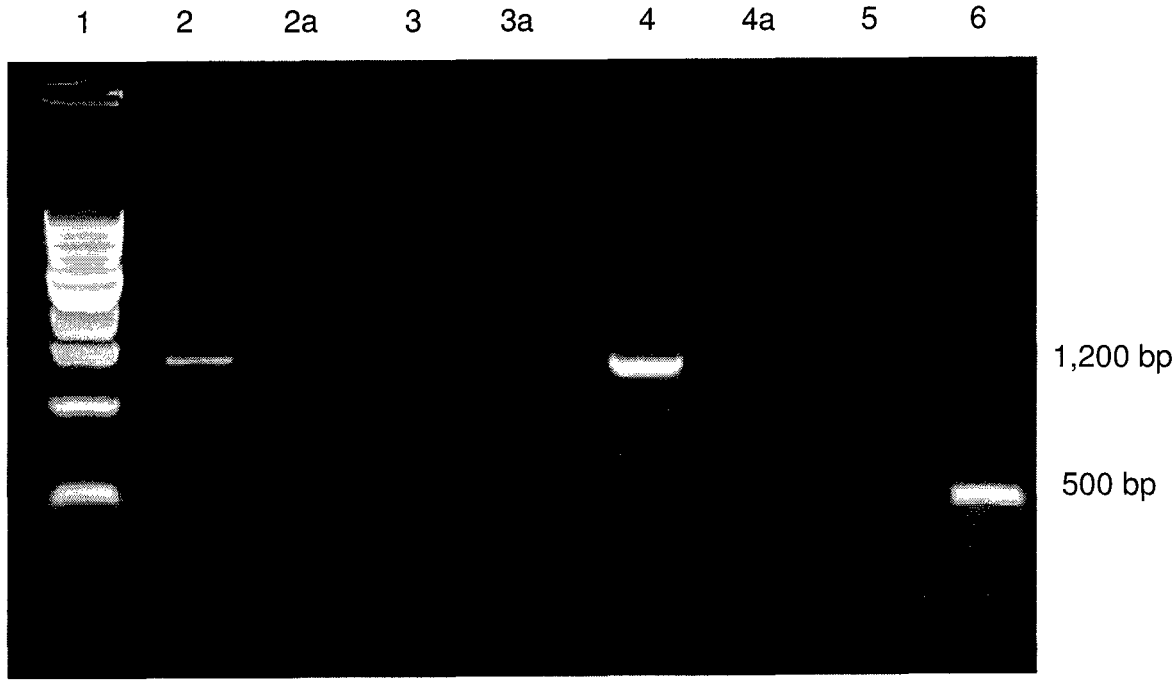
- Figure 1-

Fig 1E. Viral Yield in AdCMVCDIRESE1A Exposed Cells



- Figure 1-

Fig 1F. Expression of CD Gene in CCL-51 Breast Cancer Cells



-Figure 1-

Fig 1G. In Vitro Cytotoxicity Test

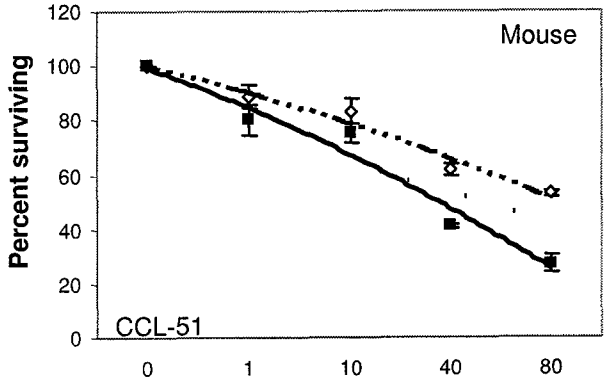


Fig 1G1

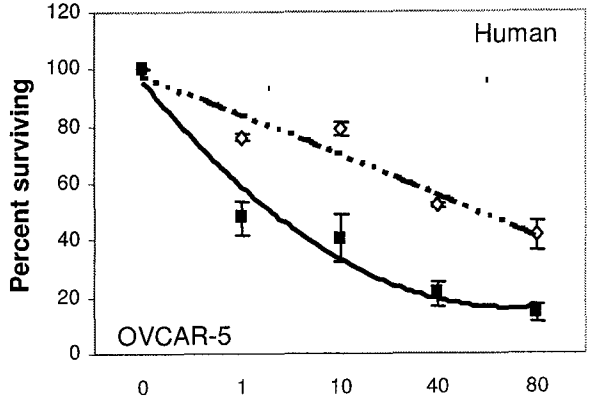


Fig 1G3

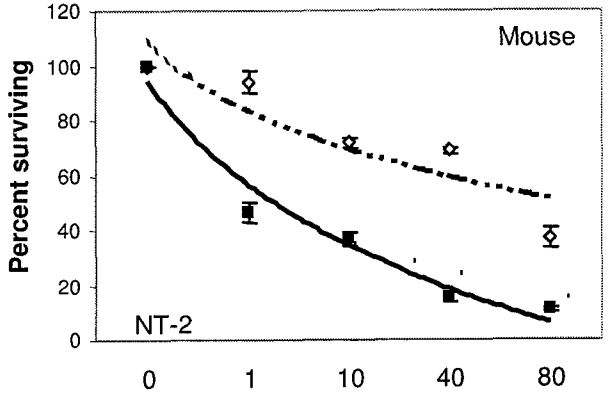


Fig 1G2

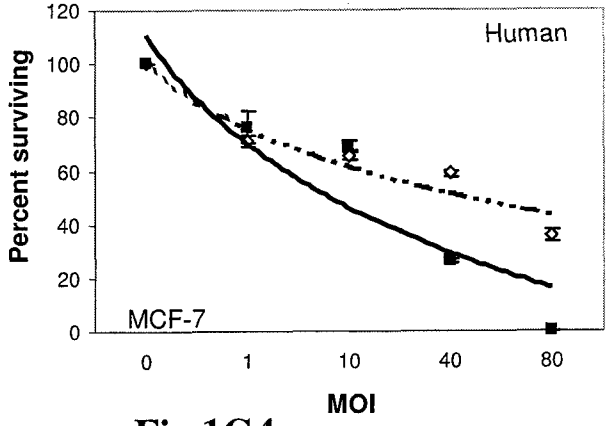
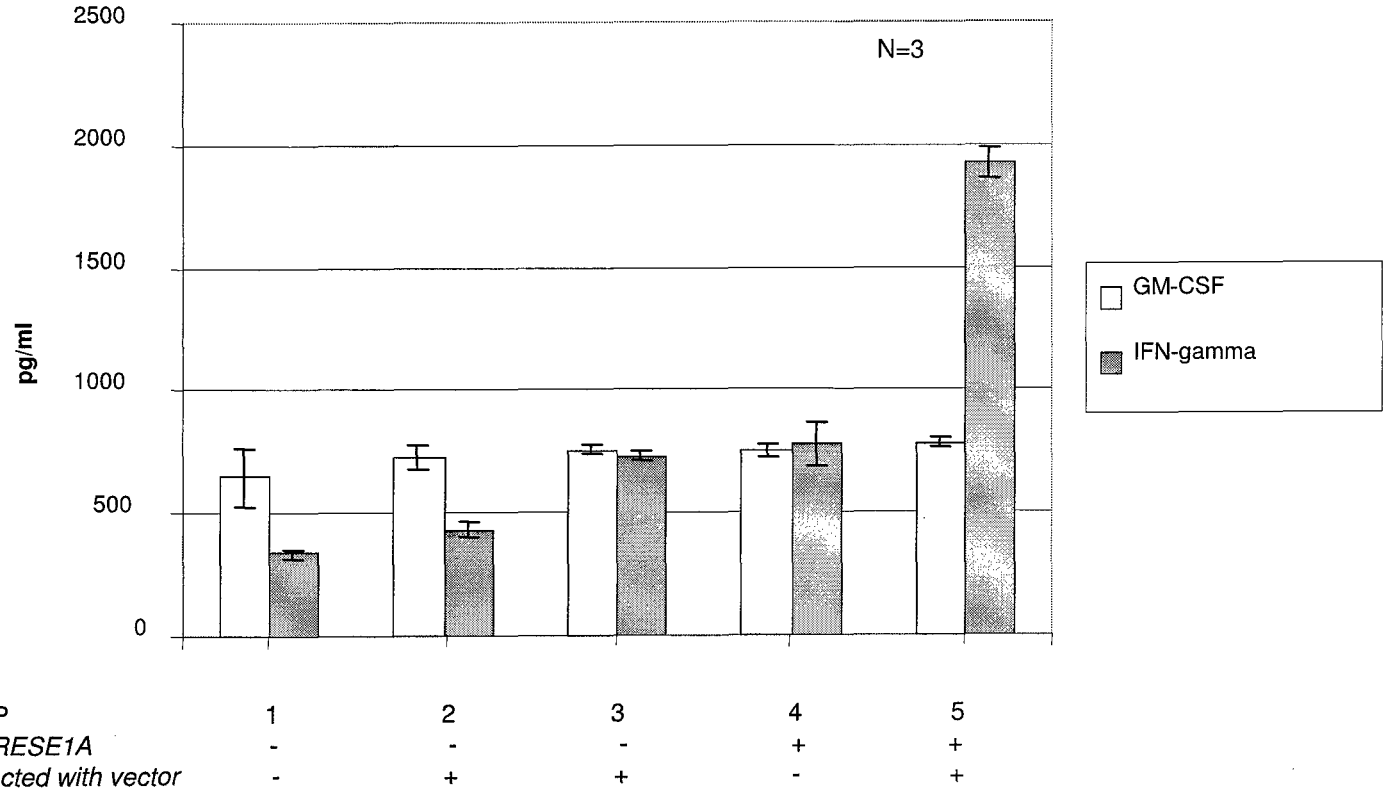


Fig 1G4

-Figure 1-

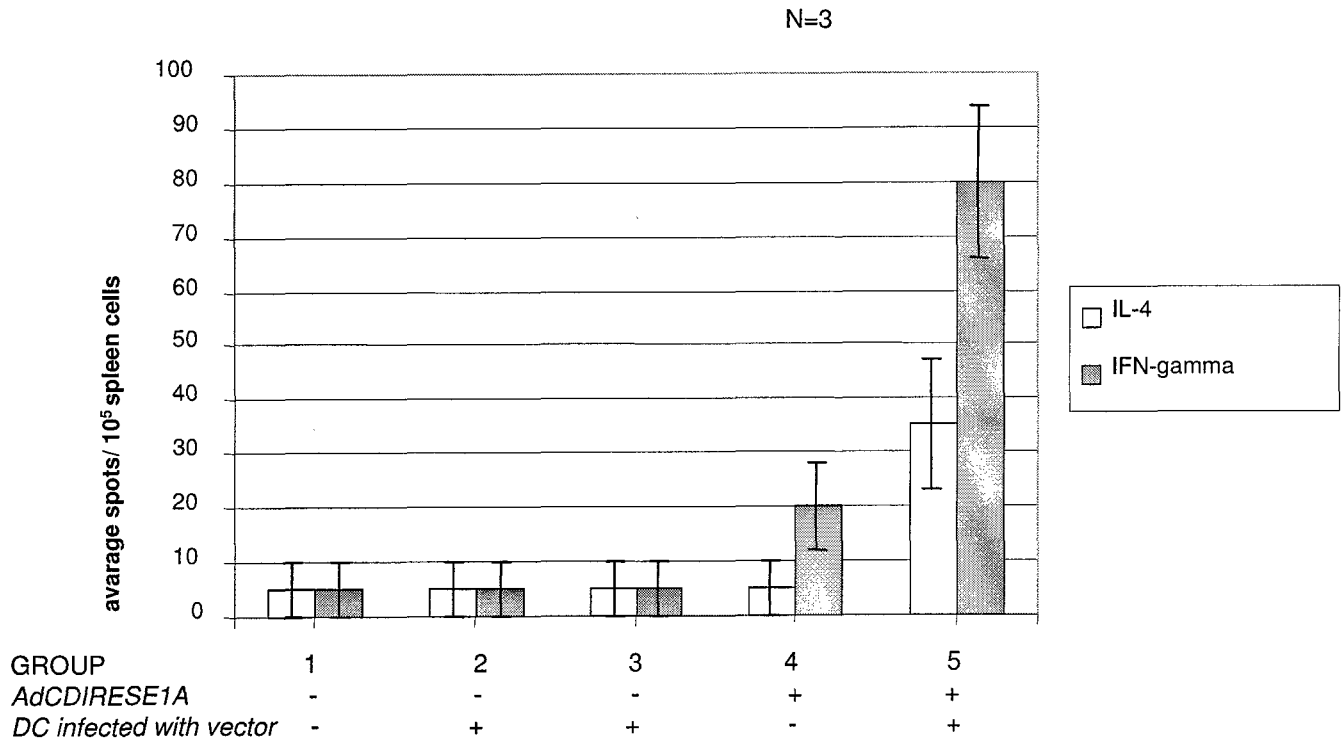
Fig 2A. Cytokine Release For Spleen Cells of Vaccinated BalbC Mice



Group #1: Control (PBS)
Group #2: DC infected with Ad-sig-GFP
Group #3: DC infected with Ad-sig-CD40L
Group #4: AdCMVCDIRESE1A+5FC only
Group #5: AdCMVCDIRESE1A+5FC + DC infected with Ad-sig-CD40L

-Figure 2-

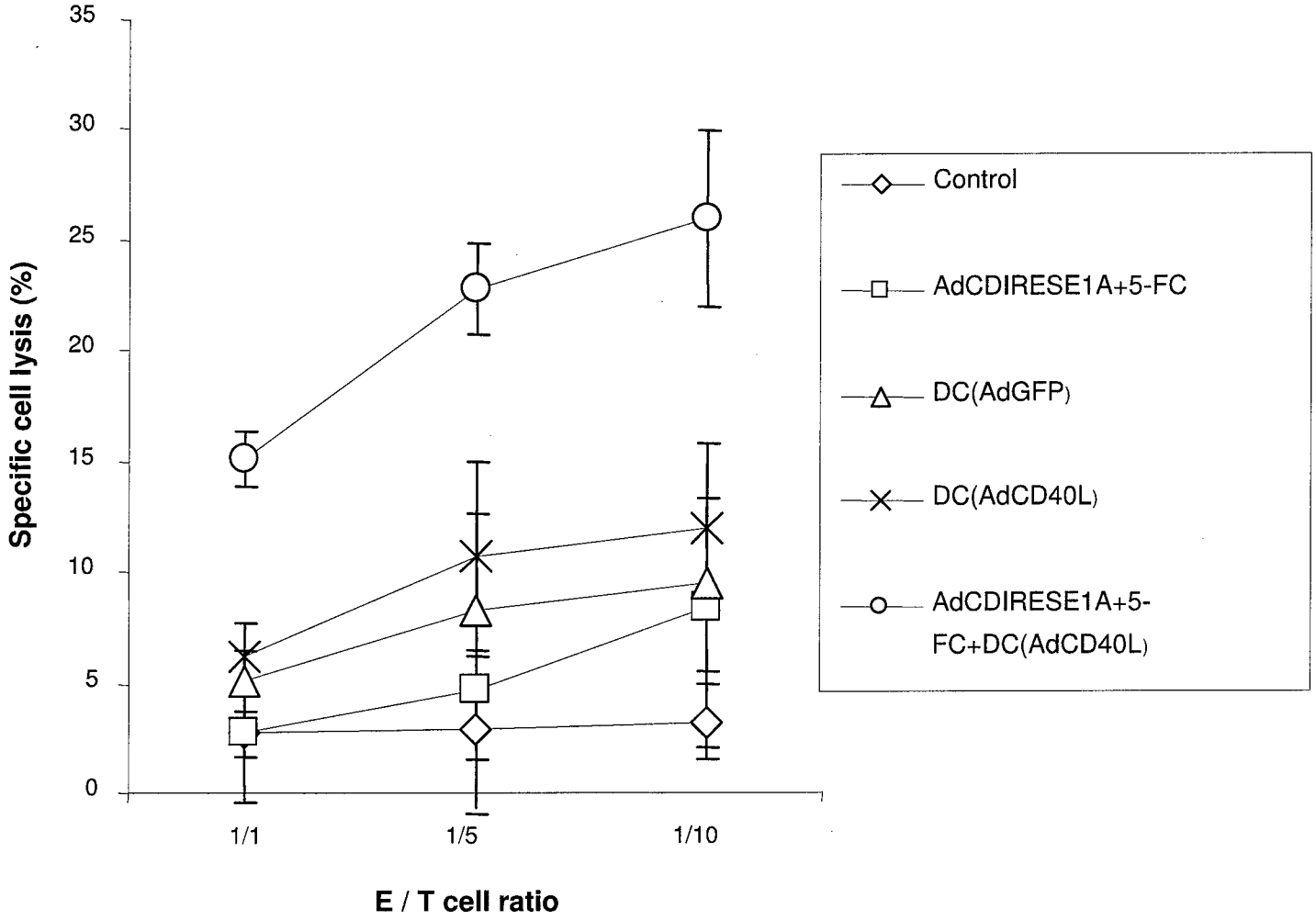
Fig 2B. ELISPOT Assay of Spleen Cells in Vaccinated BalbC Mice



Group #1: Control (PBS)
Group #2: DC infected with Ad-sig-GFP
Group #3: DC infected with Ad-sig-CD40L
Group #4: AdCMVCDIRESE1A+5FC only
Group #5: AdCMVCDIRESE1A+5FC + DC infected with Ad-sig-CD40L

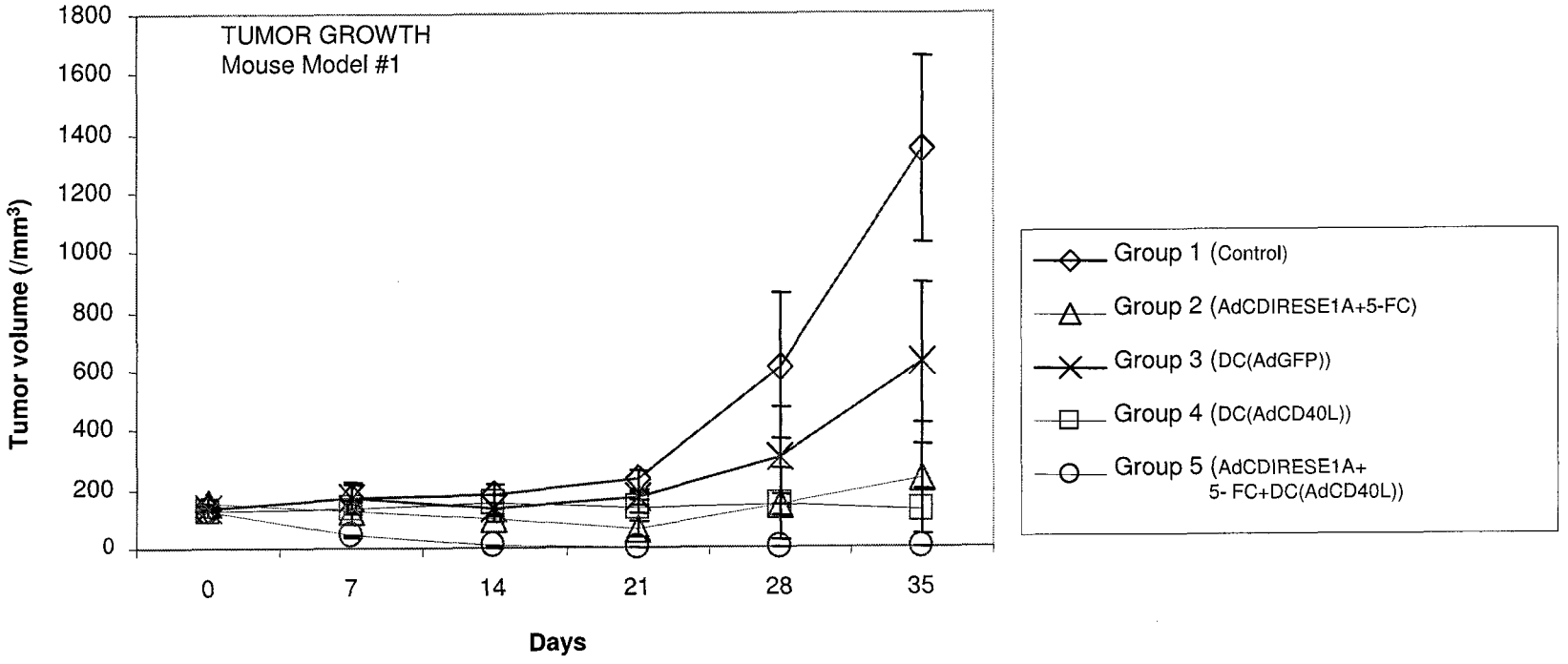
-Figure 2-

Fig 2C. Cytotoxicity Assay of Spleen Cells from Vaccinated BalbC Mice



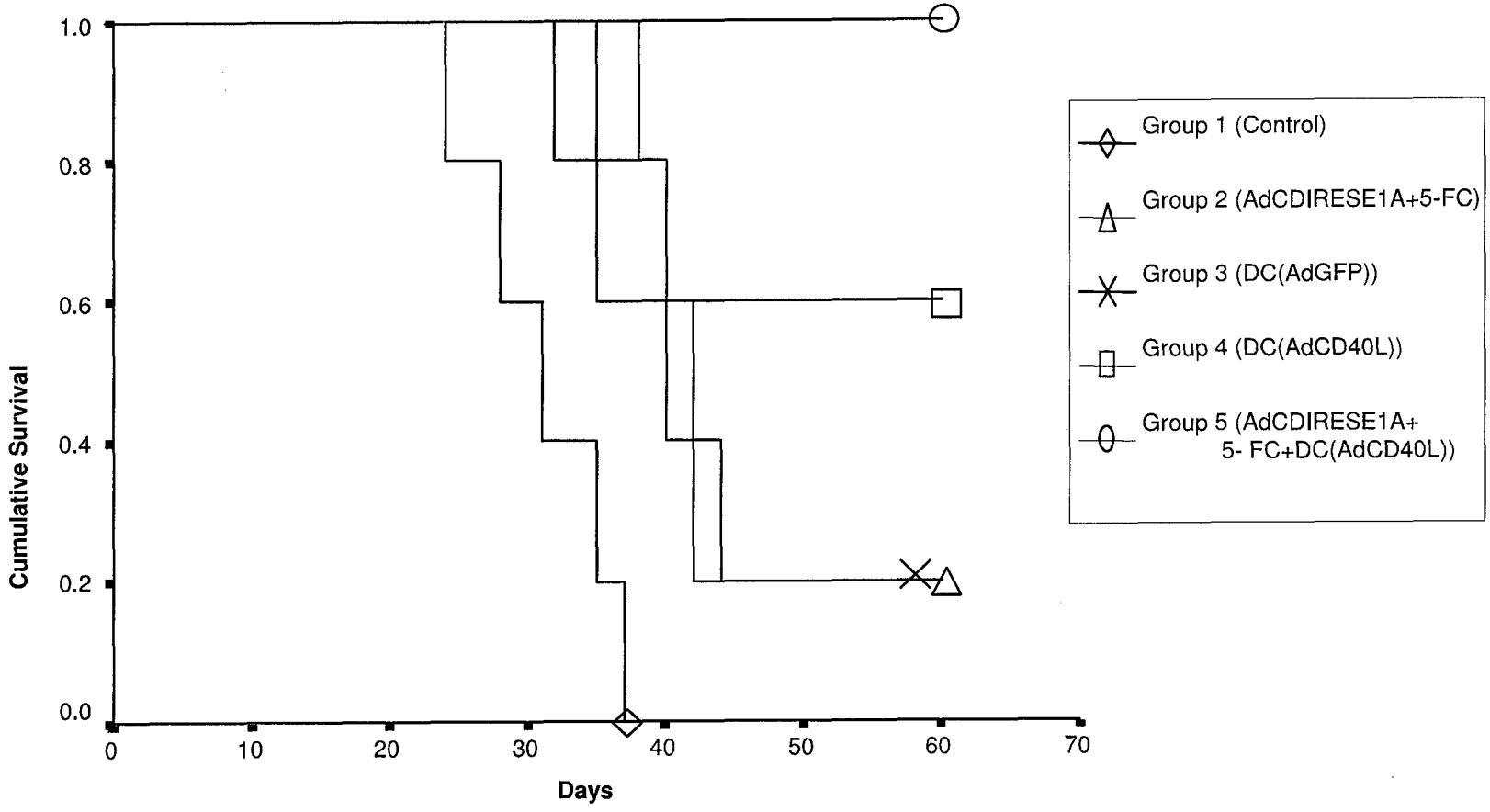
-Figure 2-

Fig 3A. Effect of Vaccine on Growth of Subcutaneous CCL-51 Tumor Nodules in BalbC Mice



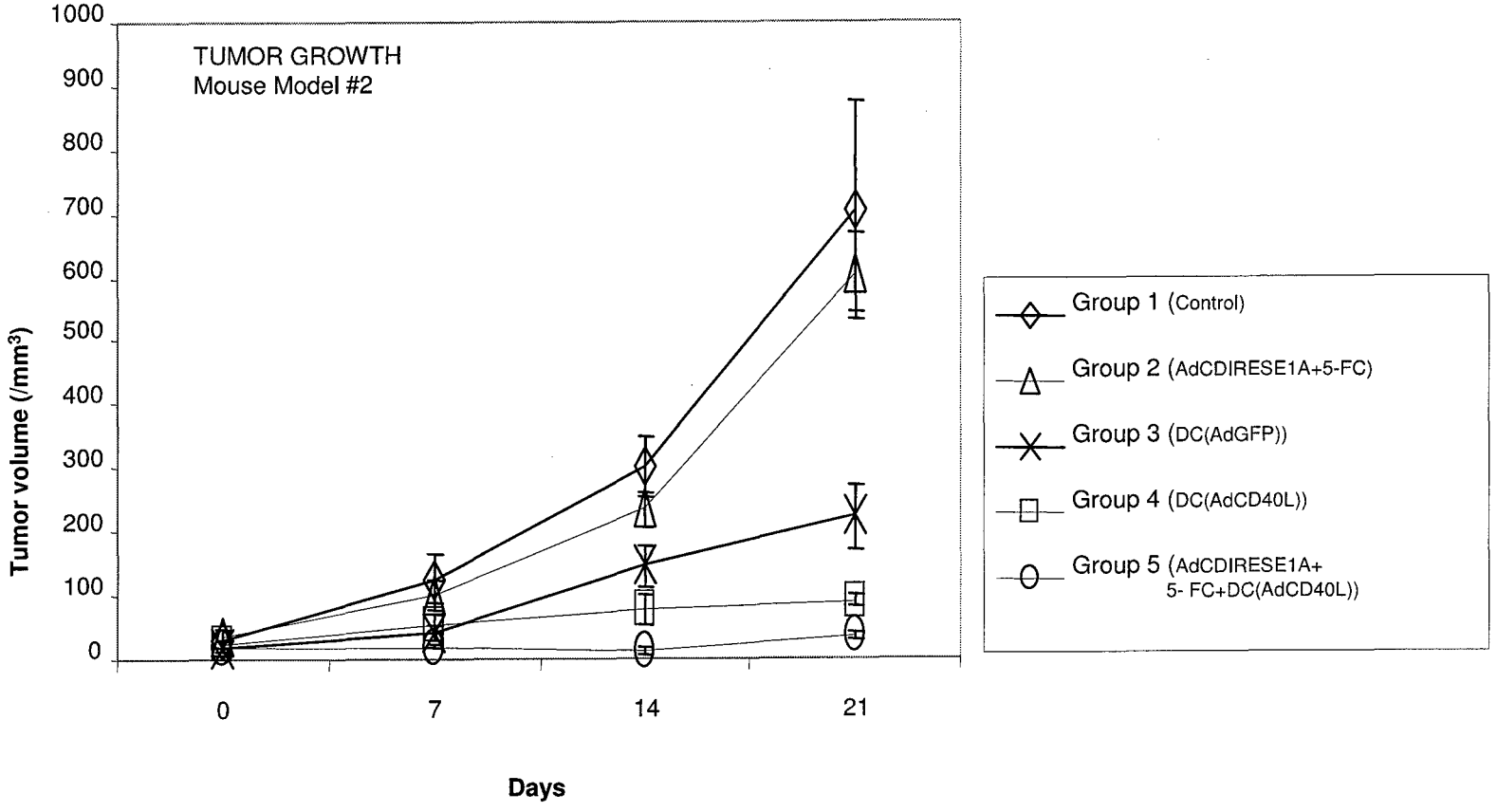
-Figure 3-

Fig 3B Survival of Vaccinated BalbC Mice Following s.c. Injection of CCL-51 Cells.



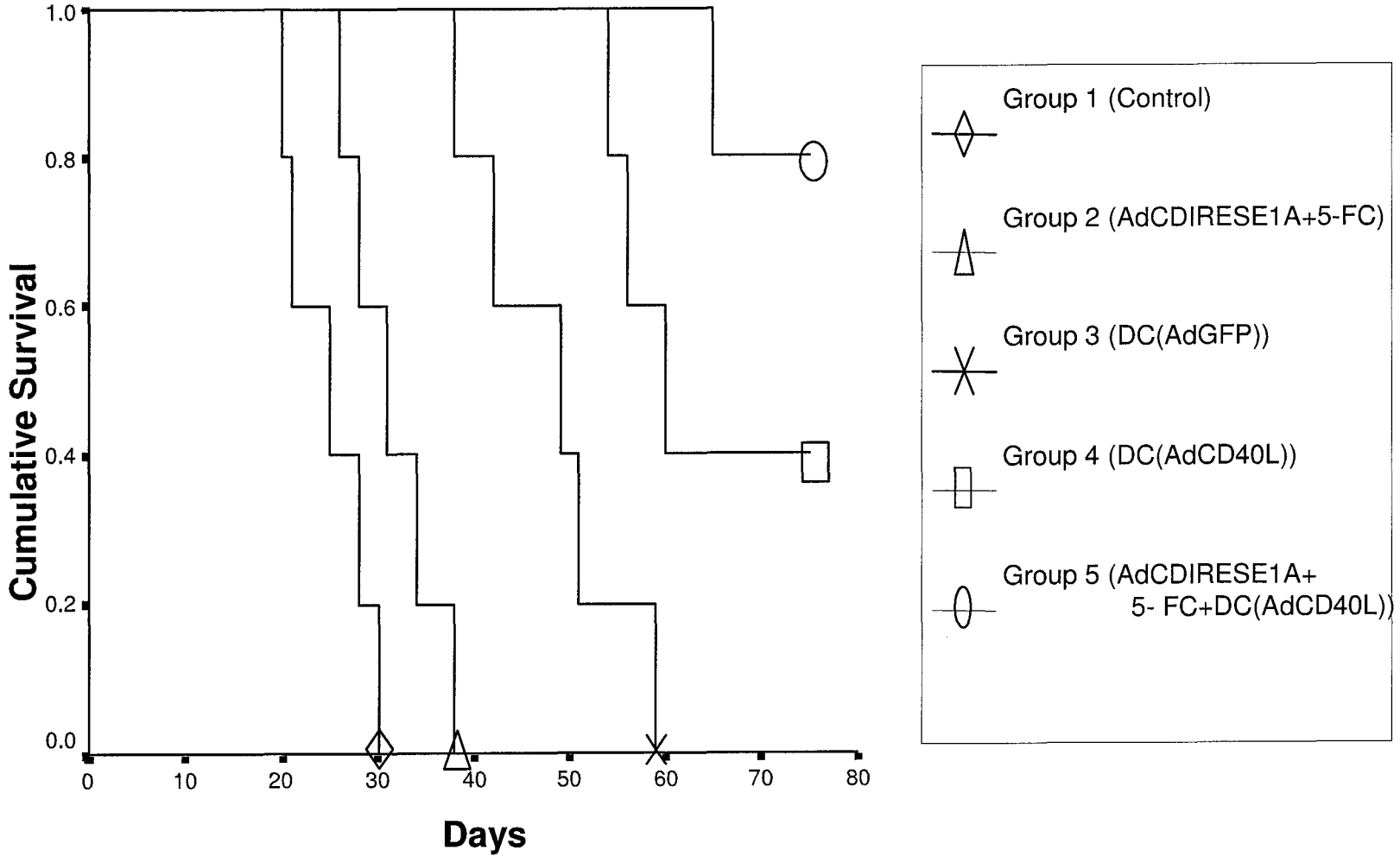
-Figure 3-

Fig 3C. Effect of Vaccination on Growth of Subcutaneous NT-2 Tumor Nodules in rH2N Transgenic Mice



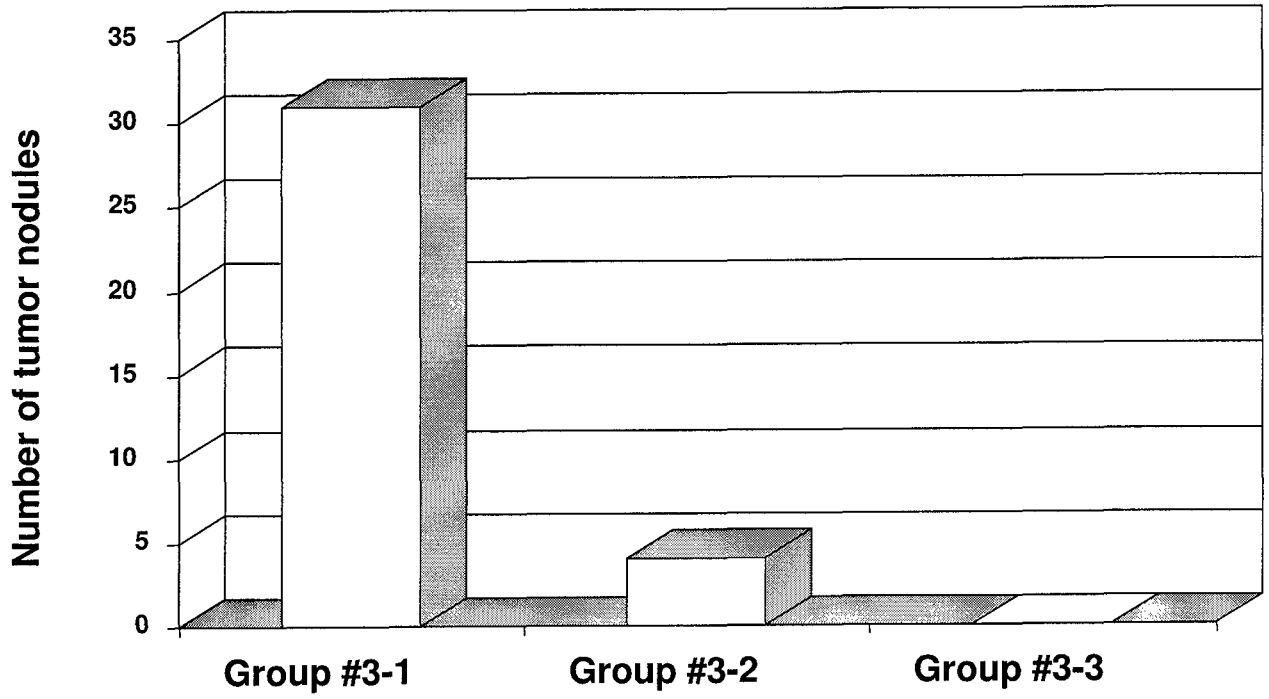
-Figure 3-

Fig 3D. Survival of Vaccinated rH2N Transgenic Mice Following s.c. Injection of NT-2 Cells



-Figure 3-

Fig 3E. Effect of Vaccine on Number of Breast Cancer Pulmonary Nodules Following IV Injection of CCL-51 Cells



-Figure 3-

Vector Prime-Protein Boost Vaccine for Breast Cancer Cells

By

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Subject Area: Cancer Vaccines
Key Words: Vaccines, Adenovirus, CD40L

Related Manuscripts:

1. Zhang, L., Tang, Y.C., Akbulut, H., Zelterman, D., Linton, P.J., and Deisseroth, A. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. Proc. Natl. Acad. Sci. USA, 100: 15202-15106, (2003).

2. Tang, Y., Zhang, L., Yuan, J., Akbulut, H., Maynard, J., Linton, P.J., and Deisseroth, A. Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens. Blood, 104: 2704-2713, (2004).

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Abstract

Background: The immune response has been shown to ignore cancer cells because most TAA are present on normal cells since birth. Even when TAA specific antibodies and CD8 effector cells are generated, they often are present at low levels, or do not penetrate the tumor tissue. We have test the Ad-sig-TAA/ecdCD40L vector prime-TAA/ecdCD40L protein boost vaccination to determine if this strategy can suppress in the vivo growth of TAA positive tumor cells.

Methods: The induction of the immune response by the TAA/ecdCD40L vector prime/protein boost strategy will be tested in TAA.Tg mice which are anergic to the TAA.

Results: We have shown that the Ad-sig-TAA/ecdCD40L vector vaccination can break tolerance in two different TAA transgenic mouse strains which are anergic for the TAA. In addition, when the TAA/ecdCD40L protein is given sc as a boost at 7 and 21 days following the sc injection of the Ad-sig-TAA/ecdCD40L vector, when the TAA were either human MUC-1 (hMUC-1) or rat Her-2-NEU (rH2N), the vaccines completely suppress the growth of TAA positive tumor cells, and dramatically increase the levels of the TAA specific T cells and antibodies over that seen with vector alone. The vaccination induced antibodies reacted with human breast cancer cells and induced the levels of CD8 effector T cells in the tumor tissue.

Conclusions: Since these TAA are upregulated in a wide spectrum of epithelial neoplasms, this vaccine is likely to reduce the rate of recurrence of these cancers following initial local therapy.

Introduction

The immune response is tolerant of many forms of cancer because cancer cells are covered by "self antigens" that have been present on normal cells from birth. In addition, antigen specific antibodies and T cells have difficulty in penetrating the extravascular tumor tissue. We have designed an Ad-sig-TAA/ecdCD40L adenoviral vector prime-TAA/ecdCD40L protein boost vaccine (see Figure 1A) for the in vivo activation and tumor associated antigen (TAA) loading of dendritic cells (DCs). Subcutaneous (sc) injection of the Ad-sig-TAA/ecdCD40L adenoviral vector (1-2) results in the secretion for 10 days of a fusion protein composed of a TAA fragment fused to the extracellular domain (ecd) of the CD40 ligand (CD40L). CD40L is a homo-trimeric

protein normally found on B cells and helper $CD4^+$ T cell lymphocytes (3-4). All of the sequences necessary to stabilize this trimeric structure of the protein are contained within the ecd of the CD40L protein (5). The binding of the TAA/ecdCD40L protein to DCs induces migration of these DCs to the regional lymph nodes (1, 6). These DCs carry fragments of TAA bound to surface MHC Class I molecules (6).

In this paper, we show that the sc injection of the Ad-sig-hMUC-1/ecdCD40L vector prime-TAA/ecdCD40L protein boost vaccine can induce a cellular and humoral immune response against the MUC-1 TAA, the overexpression of which is known to be associated with bad prognosis in human breast cancer (7-14). The MUC-1 antigen (7-9) is a structural protein expressed at very low levels on the apical surface of normal epithelial cells (see Figure 1B). The over expression of the MUC-1 protein (see Figure 1C) in carcinomas of the breast, lung, prostate, ovary, cervix, endometrium, esophagous, stomach and colon is associated with metastasis, resistance to chemotherapy, and a bad prognosis (9-14). We show that the sc injection of the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine can induce a MUC-1 specific immune response that suppresses the growth of hMUC-1 positive cancer cells in hMUC-1.Tg transgenic mice which are anergic to hMUC-1 (15). Our studies also showed that the sc injection of the hMUC-1/ecdCD40L protein at 7 and 21 days after the sc injection of the Ad-sig-hMUC-1/ecdCD40L protein dramatically increased the levels of the hMUC-1 specific CD8 effector cells and antibodies, which were shown to bind to human breast and prostate cancer cells.

We also tested if the Ad-sig-rH2N/ecdCD40L vector prime vaccine induced an immune response against the rat Her-2-Neu (rH2N) receptor, which is associated with poor prognosis in human breast cancer (16). We also showed that the sc injection of the Ad-sig-rH2N/ecdCD40L adenoviral vector into the rH2N.Tg transgenic mouse (17) induced resistance to the growth of rH2N positive cancer cells, increased the level of tumor infiltrating effector CD8 antigen specific T cells, and induced an increase in the level of expression of the *CCR5 receptor and CCL3 ligand* genes in the effector T cells, which promotes the infiltration of antigen specific effector T cells into the target tumor tissues.

These data suggest that the Ad-sig-TAA/ecdCD40L vaccine strategy which provides an adenoviral danger signal (18) along with a protein that TAA loads and activates DCs (CD40L), may be of use for suppression of recurrence of epithelial cancers after surgery and/or radiation therapy. Finally, since the Ad-sig-TAA/ecdCD40L vaccine is independent of CD4 cells, which may be functionally defective in older individuals, this vaccine may be useful for treatment of epithelial neoplasms which show a preponderance in individuals in the fifth and sixth decade of life (19).

Methods

Cell Lines.

The rH2N positive NT2 mammary tumor cell line was obtained from Dupont. The LL2/LL1hMUC-1 cell line, which was derived from LL2/LL1 (ATCC catalogue number CRL-1642), was genetically modified to express hMUC-1 by transfection with the plasmid pcDNA3-hMUC-1 and selected by growth in medium supplemented with 1mg/ml of G418.

Construction of TAA/ecdCD40L Plasmids and Vectors.

The Ad-sig-E7/ecdCD40L and the Ad-sig-ecdhMUC-1/ecdCD40L plasmid expression vectors were constructed as described previously (1-2). K/ratHer2/Neu with the upstream kappa signal sequence was generated by four rounds of PCR amplification (1^{st} round: primers 4 +5; 2^{nd} round: primer 3+5; 3^{rd} round: primer 2+5; 4^{th} round: primer 1+5). The signal peptide encoding the mouse IgG kappa chain METDTLLLWVLLLWVPG was added before Her2/Neu cDNA by PCR amplification which encodes the mouse IgG kappa chain signal sequence METDTLLLWVLLLWVPGSTGD . The primers are as follows:

The forward primer 1 is:

5'-CCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG-3' (1)

The forward primer 2 is:

5'- TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC-3' (2)

The forward primer 3 is:

5'-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC GAA CTC -3' (3)

The forward primer for the rH2N extracellular domain 4 is:

5'- TCC ACT GGT GAC CCA GAC AGT CTC CGT GAC CTC -3' (4)

The reverse primer for the rH2N extracellular domain 5 is:

5'- GGAG CTC GAG GAC CAC CAC TAA GAT CAG GAA CAG -3' (5)

The K/rH2N encoding DNA was cloned into the pcDNA™ 3.1 TOPO vector (InVitrogen, San Diego, CA) forming pcDNA-K/.rH2N. The ecd of the mouse CD40L was amplified from the template of pshuttle-hMUC1/ecdCD40L, which was inserted into the plasmid pcDNA-K/rH2N after restriction endonuclease digestion with XbaI and NotI. The primers for CD40L are: 5'-GGAAGATCTCCCAAGCTTCCTCCAGTCCACAATGTCACCCTC-3' and 5'-TTGCGGCCGCTCAGAGTTTGAGTAAGCCAAAAGATGAG-3'. The K/rH2N/ecdCD40L encoding DNA was cut from the pCDNA3TOPO vector using

HindIII-NotI restriction endonuclease digestion and inserted into the pShuttle-CMV downstream of the CMV promoter. The recombinant adenoviral vectors were generated using the AdEasy vector system (20). Briefly, the resulting plasmid pShuttle-CMV K/rH2N/ecdCD40L was linearized by PME I digestion and then co-transformed into E. coli strain BJ5183 together with pAdEasy-1 (20).

Production of hMUC-1/ecdCD40L Protein.

The hMUC1/ecdCD40L cDNA was amplified from the template pshuttle hMUC-1/ecdCD40L with the primers 5'-GGAAGATCTTCCCCACCATGGAGACAGACACACTCC–3'and 5'-TTGCGGCCGCTCAGAGTTTGAGTAAGCCAAAAGATGA G-3'. The product was inserted into the pTriEx-2 hygro Vectors (Novagen) following BglII and NotI digestion. Following incubation in IPTG supplemented medium for 4 hours, the cell lysate was prepared by the CellyticB Plus Kit (Sigma). The hMUC-1/ecdCD40L protein was purified from the soluble fraction by HIS-select Nickel Affinity Gel (Sigma). Then, the protein was concentrated and desalted by centrifugation through an Ultrafree-15 Biomax-50 filter (Millipore) and eluted with PBS.

ELISPOT Assays for Interferon-gamma Positive Antigen Specific T Cells Following Ad-sig-TAA/ecdCD40L Vector Prime-TAA/ecdCD40L Protein Boost Vaccination.

The presence of antigen specific effector T cells in the immunized mice was assessed by ELISPOT assays, as previously described (1-2).

Study of Effect of Ad-sig-rTAA/ecdCD40L Vector Prime and TAA/ecdCD40L Protein Boost in TAA Transgenic Mice.

Mice (4 per group) which were transgenic for the *rH2N* or *hMUC-1* genes (17) and therefore anergic to the cells carrying these proteins, were vaccinated via sc injection with $1X10^8$ pfu of the Ad-sig-TAA/ecdCD40L vector. One week later, mice were boosted with the same adenoviral vector injection or with a sc injection of the TAA/ecdCD40L protein at 7 and 21 days after the vector vaccination. One week after the last vaccination, TAA.Tg mice were challenged by sc injection of 5×10^5 TAA positive cancer cells/mouse. The volumes of tumor nodules were measured by caliper. The tumor volume was calculated as tumor volume=length x $(\text{width}^2)/2$, assuming an elipse. Two types of experiments were carried out: 1.) The "Prevention Experiment" in which the vaccination precedes the sc injection of the target TAA positive tumor cell line (which is summarized in Figure 1D); and 2. The "Therapy Experiment", which is summarized in Figure 1E, in which the vaccination is delivered sc following the sc injection of the TAA

positive tumor cell line. Mouse experiments were conducted under protocols approved by the SKCC IACUC.

Study of hMUC-1 and rH2N Antibody Levels Before and After Vaccination.

Blood was collected from test mice before and 1 week after the last Ad-sig-TAA/ecdCD40L or TAA/ecdCD40L sc vaccination. Serum samples were titrated for the presence of TAA specific antibody by ELISA as reported previously (1-2).

Study of Tumor Infiltrating CD8 Effector T Cell Levels in Tumor Tissue Before and After Vaccination.

We minced sc tumor nodules of rH2N.Tg mice before and after two sc injections of the Ad-sig-rH2N/ecdCD40L vector. Single cell suspensions were generated from the tumor tissue after mincing, treatment with 0.03% DNAse, treatment with 0.14% collagenase I, and filtration through Nylon mesh. FACS analysis was used to measure the level of cells in the tumor tissue with the immunophenotype of $CD8^{high}$, $CD44^{high}$, $LY6C^{high}$, and $CD62L^{low}$ which are effector T cells.

Study of the Changes of the Patterns of Gene Expression in Tumor Infiltrating Effector T Cells Following Ad-sig-TAA/ecdCD40L Prime-TAA/ecdCD40L Protein Boost Vaccination.

Tumor tissue was harvested 7 days following vaccination, minced, treated with collagenase, and strained through gauze to develop a suspension of single cells. CD8 effector T cells were purified from this population using the FACS Aria preparative cell sorter. The cells were then enriched for the following phenotype using fluorescent conjugated antibodies which recognize: $CD8^{high}$, $CD44^{high}$, $LY6C^{high}$, and $CD62L^{low}$. RNA was purified from these cells, and cDNA libraries made. We then carried out an analysis of the expression of genes which exhibited increases of over 5 fold or more following vaccination by methods described in the Affymetrix manual. Both supervised pathway analysis and unsupervised cluster analysis were carried out.

Statistical Methods.

The linear-mixed model was used for tumor growth curves and analyses with multiple time points.

Results

Ad-sig-hMUC-1/ecdCD40L Vaccine Prevents Engraftment of hMUC-1 Positive Syngeneic Cancer Cells ("Prevention Experiment").

As shown in Figure 1B, the MUC-1 protein consists of two subunits. Subunit I consists of a large extracellular protein which carries a large but variable (up to 90) number of 20 amino acid (AA) highly glycosylated repeat domains (7-8). Subunit II has a transmembrane domain with a 65 AA cytoplasmic domain, and a 69 AA extracellular domain. Subunits I and II bind to each other through non-covalent interactions. We administered the Ad-sig-hMUC-1/ecdCD40L vaccine prior to sc injection of the LL2/LL1hMUC-1 positive tumor cell line in hMUC-1.Tg mice which are anergic to hMUC-1 (15). This is called the "Prevention Experiment" (see Figure 1D). Two subcutaneous injections at 7 day intervals of the Ad-sig-hMUC-1/ecdCD40L vector into hMUC-1.Tg mice (see solid squares in Figure 2A). This vector encodes two 20 amino acid tandem repeats from an epitope of Subunit I of the hMUC-1 linked to ecd of the CD40L. As shown in Figure 2A, this confers significant resistance to the growth of the hMUC-1 positive LL2/LL1hMUC-1 cancer cell line. As shown in Figure 2B, the sc injection of the Ad-sig-hIIMUC-1/ecdCD40L vector, which encodes an epitope of Subunit II of the MUC-1 antigen (hIIMUC-1) linked to the ecd of CD40L, also induces resistance to the growth of the LL2/LL1hMUC-1 cell line in the hMUC-1.Tg mice.

Injection of the hMUC-1/ecdCD40L Protein Boosts the Immune Response Induced by the Ad-sig-hMUC-1/ecdCD40L Vector.

Several pre-clinical studies as well as clinical trials have shown that the sc injections of two different expression vectors, one as a prime and the second as a boost, expands the magnitude of the immune response induced by a vector vaccination against a specific target antigen (21-22). We therefore tested if the magnitude of the immune response induced by the Ad-sig-hMUC-1/ecdCD40L vector could be expanded by the sc injection of a hMUC-1/ecdCD40L protein which had been produced in bacterial cells.

As shown in Figure 2C, three sc injections of the hMUC-1/ecdCD40L protein (PPP) at days 0, 7, and 21 days without antecedent injection of the Ad-sig-hMUC-1/ecdCD40L vector does not completely suppress the growth of the LL2/LL1hMUC-1 tumor cell line. In contrast, the administration of three sc injections of the Ad-sig-hMUC-1/ecdCD40L vector (VVV) at 0, 7 and 21 days completely suppressed the growth of the hMUC-1 positive LL2/LL1hMUC-1 cancer cell line. As is also shown in Figure 2C, the administration of one sc Ad-sig-hMUC-1/ecdCD40L vector injection followed in 7 and

21 days by two hMUC-1/ecdCD40L sc protein injections (VPP), also induces resistance to the growth of the hMUC-1 positive cancer cell line in hMUC-1.Tg mice.

We then measured the effect of several different combinations of vector and protein boosts (see Table I). As shown in Figure 2D, the levels of hMUC-1 specific T cells in the spleens of the vaccinated animals was highest with a single Ad-sig-hMUC-1/ecdCD40L vector injection followed by two hMUC-1/ecdCD40L protein injections (T5 in Figures 2D). We will refer to this schedule of vaccination as VPP. This was 6 times as high as the level of antigen specific T cells following two vector injections (see "control" in Figure 20). The unglycosylated form of the hMUC-1/ecdCD40L protein produced in bacterial cells was used for the booster injections. As shown in Figure 2E, the VPP regimen (T5) induced levels of hMUC-1 specific antibody which were greater than any of the other combinations of vector prime and protein boost. This was the schedule of vector and protein vaccine that induced the highest level of hMUC-1 specific T cells as well (see Figure 2D).

As shown in Panel I of Figure 2F, the antibodies induced by the VPP vaccination bound to human breast cancer cells but not to the surrounding stromal cells. Exposure of the mouse serum to the specific hMUC-1 20 AA repeat peptide encoded by the vector or protein transcription units blocked completely the binding of the mouse antibodies to the breast cancer cells (see Panel II of Figure 2F). Serum from unvaccinated mice did not bind to the human breast cancer cells (see Panel III of Figure 2F). The AA sequence of the hMUC-1 peptide was then scrambled so that the order of the AA was

randomized but the composition of AA remained the same. This peptide did not block the binding of the serum from the vaccinated hMUC-1.Tg mice (data not shown).

VPP and VVV Vaccination in hMUC-1.Tg Mice with Pre-Existing Subcutaneous Tumor Nodules Induces Regression of Tumor Nodules ("Treatment Experiment").

We compared the effect of various schedules of Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost (Subunit I) in hMUC-1.Tg mice with established subcutaneous nodules of hMUC-1 positive LL2/LL1hMUC-1 cancer cells. This is called the "Treatment Experiment" (see Figure 1E). One Ad-sig-hMUC-1/ecdCD40L vector prime sc injection followed in 7 and 21 days by protein boost injections (VPP) completely suppressed the growth of the tumor cells (see solid diamonds in Figure 2G). Three vector injections (VVV) were less effective than the VPP vaccination in suppressing the growth of pre-established LL2/LL1hMUC-1 tumor cells (see solid squares in Figure 2G). In contrast, as shown in Figure 2G, three sequential sc injections of the hMUC-1/ecdCD40L protein (PPP), without antecedent vector injection, failed to completely suppress the growth of the hMUC-1 positive tumor cell line in the hMUC-1.Tg mice (see open triangles in Figure 2G).

VPP Vector Prime-Protein Boost Vaccination Suppresses Engraftment of Intravenously Administered MUC-1 Positive mLL2/LL1hMUC-1 Cancer Cells in the Lungs of hMUC-1.Tg Mice.

To mimic tumor metastases, we challenged mice by tail vein injection of hMUC-1 positive LL2/LL1hMUC-1 tumor cells following completion of the vaccinations in the "Prevention Experiment" outlined in Figure 1D, or the "Treatment Experiment" outlined in Figure 1E. Intravenous administration of LL2/LL1hMUC-1 cells generates tumor nodules in the lungs. We then weighed the lungs of mice sacrificed 63 days following the initiation of vaccination. As shown in Figure 2H ("Prevention" side), the weight of the lungs in mice injected with PPP was 2.5 times the weight of the lungs in mice not injected intravenously with the LL2/LL1hMUC-1 cell line. In contrast, the weight of the lungs in mice injected sc with three successive Ad-sig-hMUC-1/ecdCD40L vector injections (see VVV on "Prevention" side in Figure H), or the single Ad-sig-hMUC-1/ecdCD40L vector sc injection followed by two successive sc injections of the hMUC-1/ecdCD40L protein at 7 and 21 days (VPP), was within the margin of error of the weight of the lungs in mice not injected intravenously with the LL2/LL1hMUC-1 cancer cells (see left hand side of Figure 2H).

Next, we measured the lungs of the animals which were vaccinated according to the "Treatment Experiment" plan outlined in Figure 1E in which the sc injection of the LL2/LL1hMUC-1 occurs first followed by sc vaccination and then intravenous injection of the LL2/LL2hMUC-1 cells. In this model, the sc tumor nodules are already growing by the time of the vaccination. Because the subcutaneous tumor nodules were growing to an advanced level after 2 weeks, some of the animals were sacrificed at 2 weeks earlier than in the prevention experiment. As shown in the right hand side of Figure 2H ("Treatment Experiment"), the VPP schedule of vaccination completely suppressed the

growth of the tumor cells. Furthermore, these data showed that the administration of 3 sc injections of the Ad-sig-hMUC-1/ecdCD40L vector (VVV) generates less of an immune response than did the single vector injection followed by two hMUC-1/ecdCD40L injections (VPP).

Both the MUC-1 Antigen and the CD40L Are Required To Boost the Immune Response Induced by the Ad-sig-hMUC-1/ecdCD40L Vector.

As shown in Figure 2I, the growth of hMUC-1 positive LL2/LL1 tumor cells in the hMUC-1.Tg mice was not completely suppressed by subcutaneous injection of the Ad-sig-hMUC-1/ecdCD40L vector (Subunit I) followed by the hMUC-1 Subunit I antigenic peptide linked to the KLH stabilizing molecule (hMUC-1/KLH) with or without incomplete Freund's adjuvant, or an extract of the bacterial host strain used to produce the hMUC-1/ecdCD40L as a control. In contrast, the sc injection of the As-sig-hMUC-1/ecdCD40L vector followed by the sc injection of the hMUC-1/ecdCD40L protein (see solid circles in Figure 2I) completely suppressed the growth of the hMCU-1 positive tumor cells.

The Ad-sig-rH2N/ecdCD40L Vaccine Induces rH2N Specific Immunity in rH2N.Tg Mice.

Two sc injections of the Ad-sig-rH2N/ecdCD40L vector one or two times at 7 day intervals in rH2N.Tg mice (17), which are anergic to rH2N induced complete resistance to the growth of the rH2N positive mouse N202 breast cancer cells (solid squares in Figure 3A), whereas one injection (open triangles in Figure 3A) partially suppressed the growth. As shown in Figure 3B, rH2N specific antibody levels were higher following two sc injections (solid squares) than following a single sc injection (solid circles) of the Ad-sig-rH2N/ecdCD40L vector. As shown in Figure 3C, two sc injections of the Ad-sig-rH2N/ecdCD40L vector induced levels of rH2N specific T cells in the spleens of vaccinated mice that were 10 times higher than the levels of rH2N specific T cells induced in unvaccinated mice.

Level of CD8 T Cells Infiltrating the Tumor Tissue Increased After Vaccination with the Ad-sig-rH2N/ecdCD40L Vector.

One of the predictions which could be made on the basis of previous work (23-25) is that the levels of CD8 effector T cells in the tumor tissue will be increased following vaccination with the Ad-sig-rH2N/ecdCD40L vector. We found that the number of T cells with the immunophenotype of effector T cells ($CD8^{high}$, $CD44^{high}$,

LY6C^{high}, and CD62L^{low}) isolated from the tumor tissue after vaccination was increased four fold as shown in Figure 4. This data suggest that the suppression of the growth of the rH2N positive tumor cells in the rH2N.Tg mice following Ad-sig-rH2N/ecdCD40L vaccination is mediated in part by an increase in the trafficking of effector T cells into the tumor tissue.

Changes in Gene Expression in Effector CD8 T Cells Which Infiltrate Tumor Tissue Following Vaccination.

RNA was isolated from the tumor infiltrating CD8 effector T cells was purified from the tumor tissue of vaccinated mice. The pattern of gene expression of 21 known chemokine receptors and ligands in the effector T cells which were infiltrating the tumor tissue. The chemokine pathway plays a major role in the trafficking of effector T cells from the lymph nodes draining sites of vaccination to the tissue sites harboring inflammation or infection (26-27). We observed that in the CD8 effector cells infiltrating tumor tissue, the levels of the CCL3 gene expression was increased 2.8 fold and the levels of the CCR5 were increased 16 fold. These two chemokine proteins are involved in the targeting of T cells to the extravascular sites of tissue inflammation.

Discussion

We have successfully used two transgenic mouse models in which anergy to the TAA (MUC-1 and rH2N) exists to show that the sc injection of the Ad-sig-TAA/ecdCD40L vector induces a cellular and humoral immune response which suppresses the growth of the tumor cells. The TAA choses for study are ones which are known to be associated with poor response to therapy and adverse prognosis in women with breast cancer and other common epithelial neoplasis such as carcinomas of the lung, colon, prostate and ovary (7-17). The addition of TAA/ecdCD40L protein booster sc injections following the Ad-sig-TAA/ecdCD40L vector further increased the level of antigen specific T cells and antibodies induced by the vector vaccination, whereas TAA/ecdCD40L protein given without antecedent vector injection is less effective, perhaps due to the "danger signal" of the vector (18). Important, the antibodies induced in the hMUC-1.Tg mice with the Ad-sig-hMUC-1/ecdCD40L vector prime/hMUC-1/ecdCD40L protein boost were shown to bind to human breast cancer cells in clinical biopsy material, suggesting that this cancer vaccine strategy will work human subjects with breast cancer.

One of the most challenging aspects of activating and maintaining an immune response against cancer cells, is the barrier that must be overcome to deliver the antigen specific antibodies and T cells to the tumor cells into the extravascular space. The experimental results presented in Figure 4 show that the levels of effector T cells in the tumor tissues are increased 3 fold following the Ad-sig-hMUC-1/ecdCD40L vector

injection and that these cells are programmed to attract additional T cells bearing increased expression of the CCL3 and CCR5 chemokines into the tumor tissue.

The experimental results reported in this paper suggest that the Ad-sig-TAA/ecdCD40L adenoviral vector induces an immune response that is more forceful than previous studies involving bacterial cells to deliver the TAA/ecdCD40L gene (28), since the oral DNA vaccine required an IL-2 cytokine boost, and that the Ad-sig-TAA/ecdCD40L-TAA vector prime-ecdCD40L protein boost vaccine could be used to prevent recurrence of epithelial cancer following surgery in high risk patients. Preclinical studies are ongoing to prepare for a phase I clinical trial to assess the toxicity of the Ad-sig-TAA/ecdCD40L vector prime/TAA/CD40L protein boost strategy in individuals with breast cancer as well as other epithelial neoplasms.

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Figure Legends

Figure 1: Biology of the MUC-1 Antigen in Normal and Neoplastic Epithelial Cells.

Panel A: Strategy for Ad-sig-hMUC-1/ecdCD40L Vector Vaccination: sc injection of the vector leads to the secretion from vector infected cells of the hMUC-1/ecdCD40L protein. This protein activates and hMUC-1 loads DCs which then mature and migrate to the regional lymph nodes where they stimulate hMUC-1 specific naïve CD8 cells to mature to effector cells. These cells then go out into the blood stream eventually to end up at the site of the original hMUC-1 tumor nodule.

Panel B: MUC-1 Expression in Normal Epithelial Cells. Two subunits of MUC-1 are detectable on the apical surface of normal epithelial cells. Subunit I is totally extracellular and associated through non-covalent interactions with Subunit II which has a transmembrane domain, a cytoplasmic domain and an extracellular domain.

Panel C: MUC-1 Expression in Neoplastic Epithelial Cells. MUC-1 exhibits increased levels of expression and is distributed on all surfaces of neoplastic epithelial cells. The extracellular subunit (Subunit I) is associated only on a minority of the Subunit II subunit molecule which is embedded in the membrane on the neoplastic epithelial cells.

Panel D: "Prevention" Vaccination. The administration of the sc vaccination precedes by 7 days the sc injection of the TAA positive cancer cell line into the test mice.

Panel E: "Treatment" Vaccination. The sc injection of the TAA positive cancer cell line into the test mice precedes the administration of the vaccination by 5-7 days

Figure 2A: Ad-sig-hMUC-1/ecdCD40L Vector Vaccine Which Encodes Epitope for Subunit I (All Extracellular) of the hMUC-1 Linked to the ECD of the CD40L

Suppresses Growth of the LL2/LL1hMUC-1 Cell Line in hMUC-1.Tg Mice. Test mice were injected sc with the Ad-sig-hMUC-1/ecdCD40L vector prime and hMUC-1/ecdCD40L protein boost vaccine (epitope of Subunit I of hMUC-1 linked to CD40L) and then injected sc with the LL2/LL1hMUC-1 tumor cells ("Prevention" Experiment-see Figure 1D). We then measured the size of the sc nodule which developed at the sc injection site of 500,000 LL2/LL1hMUC-1 tumor cells in hMUC-1.Tg mice which had been vaccinated with the Ad-sig-hMUC-1/ecdCD40L vector. This vector contains a 40 amino acid epitope from Subunit I of hMUC-1, which is totally extracellular. The following was used for the vaccination: No vaccination (solid diamonds); Ad-sig-hMUC-1/ecdCD40L Subunit I vector (solid squares); Ad-sig-hMUC-1 Subunit I vector (solid triangles).

Figure 2B: Ad-sig-hIIMUC-1/ecdCD40L Vector Vaccine Which Encodes Epitope for Subunit II (The Subunit Embedded in the Membrane) of hMUC-1 Linked to ECD of CD40L Suppresses Growth of the LL2/LL1hMUC-1 Cell Line in hMUC-1.Tg Mice. Test mice were injected sc with the Ad-sig-hMUC-1/ecdCD40L vector prime and hMUC-1/ecdCD40L protein boost vaccine (epitope of Subunit II of hMUC-1 linked to CD40L) and then injected sc with the LL2/LL1hMUC-1 tumor cells ("Prevention" Experiment-see Figure 1D). We then measured the size of the sc nodule which developed at the sc injection site of 500,000 LL2/LL1hMUC-1 Tumor Cells in hMUC-1.Tg mice which had been vaccinated with the Ad-sig-hMUC-1/ecdCD40L Subunit II vector. This vector contains an epitope from the ecd of Subunit II of hMUC-1. Subunit II is the subunit in Figure 1B-C which is a transmembrane protein with both an

ecd and a cytoplasmic domain. The following was used for the vaccination: Nothing (solid diamonds); Ad-sig-hMUC-1/ecdCD40L Subunit II vector (solid squares).

Figure 2C: Effect of the VVV, VPP and PPP Vaccination on the Growth of Subcutaneous Nodules of hMUC-1 Positive LL2/LL1hMUC-1 Cancer Cells When the SC Injection of the Ad-sig-hMUC-1/ecdCD40L Vector Encoding Subunit I of hMUC-1 Linked to ECD of CD40L Precedes the Subcutaneous Injection of the LL2/LL1hMUC-1 Cancer Cells ("Prevention" Experiment). The growth of sc nodules of hMUC-1 positive LL2/LL1hMUC-1 cancer cells as sc nodules was measured in hMUC-1.Tg mice, which had been injected sc with 500,000 hMUC-1 positive LL2/LL1hMUC-1 cancer cells after administration of one of the following vaccination schedules: VVV (solid triangles), VPP (solid squares) or PPP (solid diamonds). V=Ad-sig-hMUC-1/ecdCD40L Subunit I vector; P=hMUC-1/ecdCD40L Subunit I protein.

Figure 2D: The Effect of Various Schedules of the Ad-sig-hMUC-1/ecdCD40L Vector and the hMUC-1/ecdCD40L Protein (Subunit I) on the Level of Interferon-Gamma Positive T Cells in the Spleen of hMUC-1.Tg Mice Before and After Vaccination. The following combinations of the Ad-sig-hMUC-1/ecdCD40L Subunit I vector (V) and hMUC-1/ecdCD40L Subunit I protein (P) were injected sc into the hMUC-1.Tg mice: Control=VVNN; T1=VVPN; T2=VVNP; T3=VPNN; T4=VNPN; T5=VPNP; Negative Control=NNNN. N=nothing. All injections (A, P, or N) are separated at 7 day intervals. V=Vector; P=Protein; N= Nothing.

Figure 2E: The Effect of Various Schedules of the SC Injection of the Ad-sig-hMUC-1/ecdCD40L Subunit I Vector and the hMUC-1/ecdCD40L Subunit I Protein on the Level of hMUC-1 Specific Antibodies in hMUC-1.Tg Mice. The

following combinations of the Ad-sig-hMUC-1/ecdCD40L Subunit I vector (V) and hMUC-1/ecdCD40L Subunit I protein (P) were injected sc in the hMUC-1.Tg mice: Control=VVNN (solid circles); T1=VVPN (solid diamonds); T2=VVNP (solid squares); T3=VPNN (open circles); T4=VNPN (solid triangles); T5=VPNP (open squares); Negative Control=NNNN (--+--). N=nothing. All injections are at 7 day intervals. V=Ad-sig-hMUC-1/ecdCD40L Subunit I vector; P=hMUC-1/ecdCD40L Subunit I protein.

Figure 2F: Binding of Antibodies from the Serum of Ad-sig-hMUC-1/ecdCD40L Subunit I Vector Vaccinated Mice to Human Breast Cancer Cells. Serum collected from hMUC-1.Tg mice following vaccination with the Ad-sig-hMUC-1/ecdCD40L vector prime and hMUC-1/ecdCD40L protein boost (Subunit I) was applied to sections from human breast cancer clinical specimens. Panel I: Antibodies from vaccinated mice. Panel II: Antibodies from vaccinated mice which were exposed to the hMUC-1 specific peptide used in the vaccination prior to applying the mouse serum to the sections; Panel C: Serum from unvaccinated mice.

Figure 2G: Effect of the VVV, VPP and PPP Vaccination on the Growth of SC Nodules of the LL2/LL1hMUC-1 Cancer Cells When the SC Injection of the LL2/LL1hMUC-1 Cells Precedes the Ad-sig-hMUC-1/ecdCD40L Subunit I Vector Vaccination ("Treatment" Experiment-see Figure 1E). The growth of sc nodules of hMUC-1 positive LL2/LL1hMUC-1 cancer cells was measured in hMUC-1.Tg mice which were injected sc with the hMUC-1 positive LL2/LL1hMUC-1 cells 3 days prior to being vaccinated with one of the following regimens: VVV (solid squares), VPP (solid diamonds) or PPP (open triangles). V=Ad-sig-hMUC-1/ecdCD40L Subunit I vector. P=hMUC-1/ecdCD40L Subunit I protein.

Figure 2H: The Effect of Ad-sig-hMUC-1/ecdCD40L Subunit I Vector Vaccination on the Growth of the hMUC-1 Positive LL2/LL1hMUC-1 Cancer Cells in the Lungs of hMUC-1.Tg Mice. We weighed the lungs of hMUC-1.Tg mice which were vaccinated before the sc and intravenous injection of the hMUC-1 positive LL2/LL1hMUC-1 cancer cells (left hand panel-"Prevention") or in mice vaccinated after the sc injection of the LL2/LL1hMUC-1 cancer cells (right hand panel-"Treatment").

Figure 2I: Testing of Boosting Proteins Composed of hMUC-1 TAA Without CD40L. In order to test the importance of the presence of both the CD40L and the hMUC-1 to the boosting of the immune response, induced by the Ad-sig-hMUC-1/ecdCD40L vector (Subunit I), we compared the effect of the following boosts with the hMUC-1/ecdCD40L protein (Solid circles): bacterial cell lysate (Open diamonds), Keyhole Limpet Hemocyanin (KLH) conjugated hMUC-1 antigen with (Open circles) and without (Open triangles) incomplete Freund's adjuvant, and PBS (Open squares)

Figure 3A: Effect of the Ad-sig-rH2N/ecdCD40L Vector on the Growth of rH2N Positive NT2 Cells. The following vectors were injected sc twice at a 7 day interval: No vector (solid diamonds); two sc injections of the Ad-rH2N/ecdCD40L vector (solid squares), and one sc injection of the Ad-rH2N/ecdCD40L vector (open triangles).

Figure 3B: Effect of the Ad-sig-rH2N/ecdCD40L Vector on the Induction of rH2N Specific Antibodies Against rH2N Positive NT2 Cells. The following vectors were injected sc twice at a 7 day interval: No vector (solid diamonds); two sc injections of the Ad-rH2N/ecdCD40L vector (solid squares), and one sc injection of the Ad-rH2N/ecdCD40L vector (solid circles).

Figure 3C: Effect of the Ad-sig-rH2N/ecdCD40L Vector on the Induction of rH2N Specific T Cells as Measured by the ELISPOT Assay. The ELISPOT assay was used to measure the level of the interferon gamma positive T cells/1 X 10^5 spleen cells following in vitro exposure to MMC treated rH2N tumor cell lines. The T cells were collected from the spleens of mice before and after vaccination two sc injections of the Ad-sig-rH2N/ecdCD40L vector or no vaccination (control).

Figure 4: Levels of Tumor Infiltrating Effector CD8 T Cells Following Vaccination. The tumor nodules were excised, minced, filtered and then the level of the $CD44^{high}$, $CD8^{high}$, $LY6C^{high}$, and $CD62L^{low}$ surface markers were determined using the FACS Calibur.

Figure 1A Schematic of Vaccination

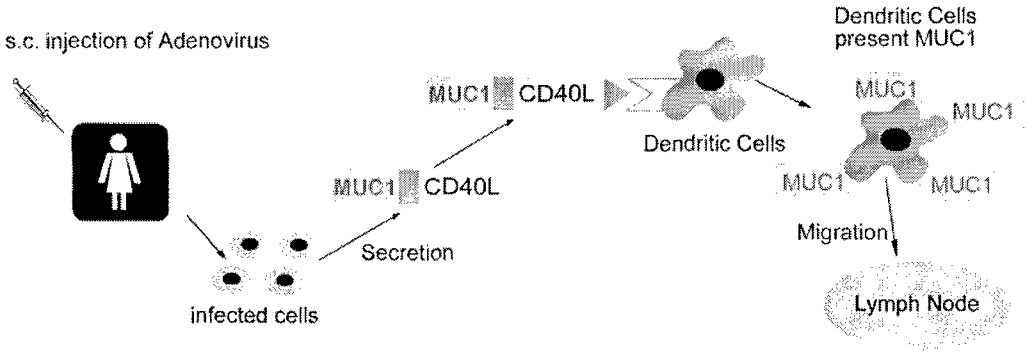


Figure 1B

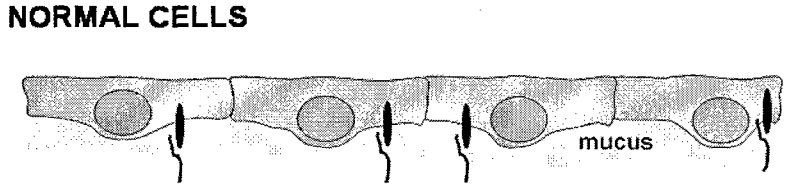


Figure 1C

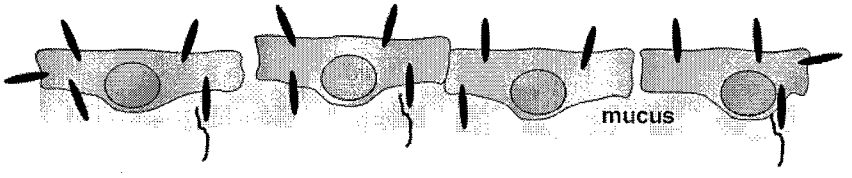


Figure 1D: Prevention

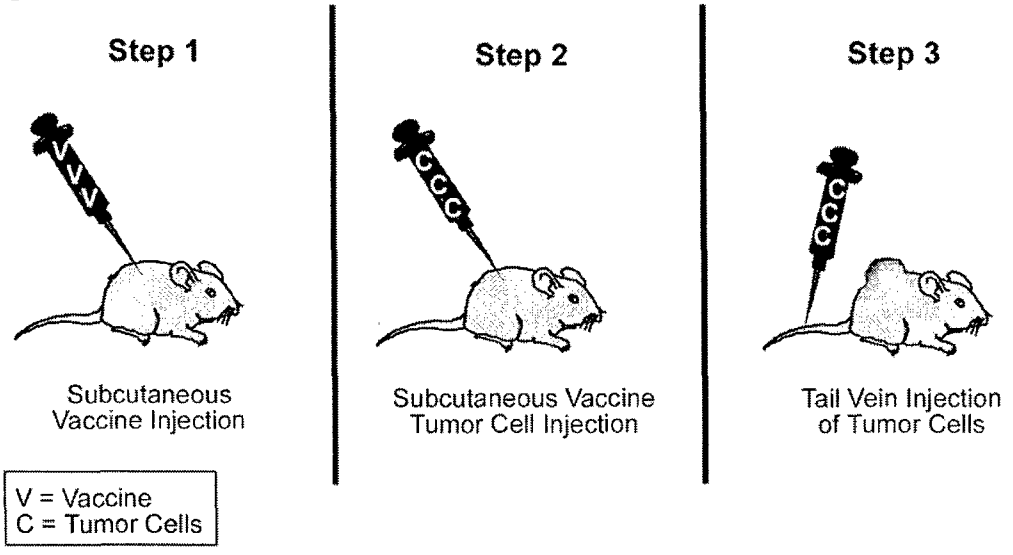


Figure 1E: Treatment

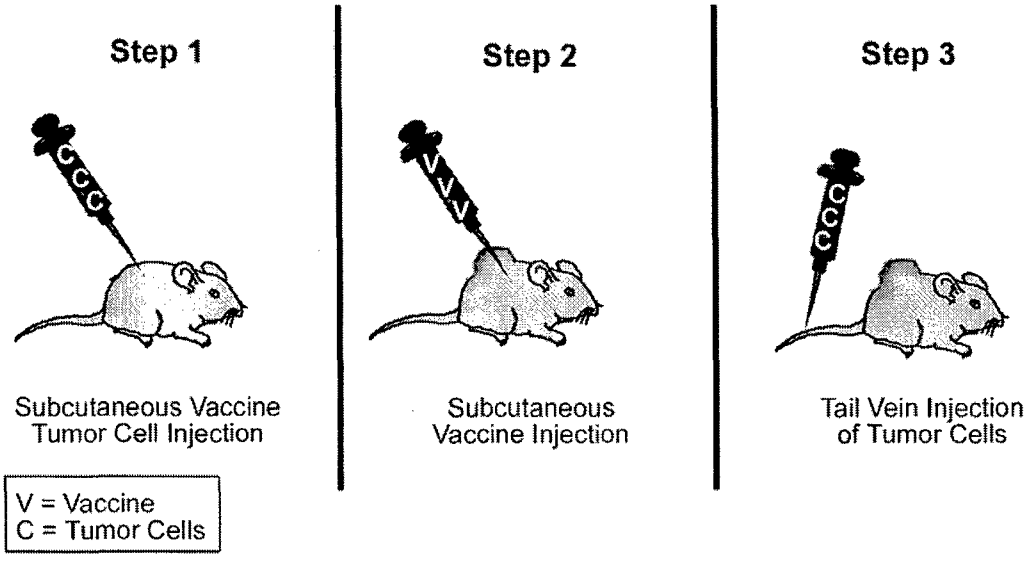


Figure 2A: Tumor Growth in hMUC-1.Tg Mice Vaccinated with Ad-sig-hMUC-1/ecdCD40L Subunit I Vaccine

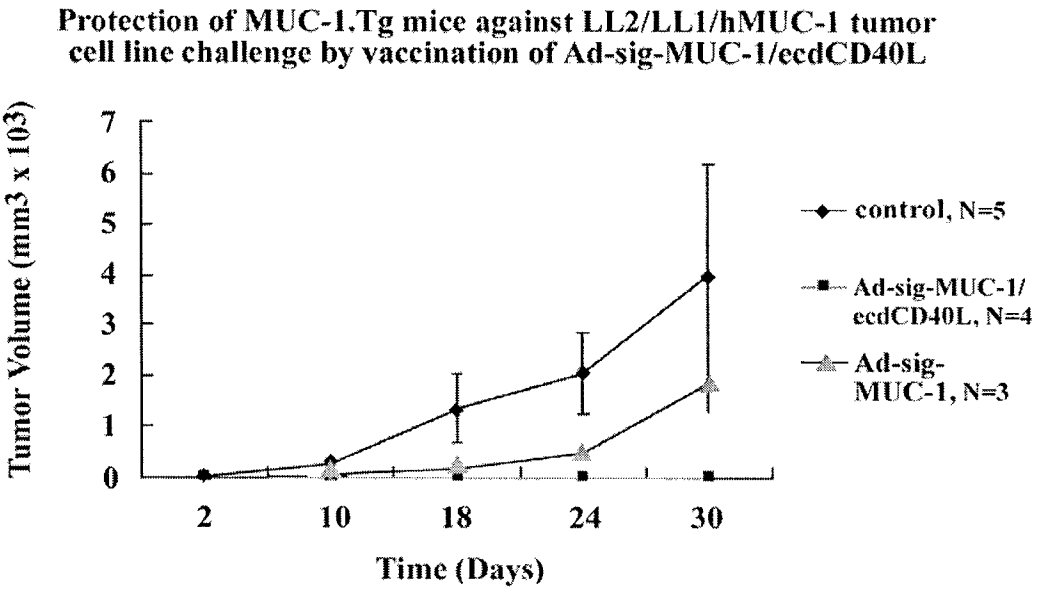


Figure 2B: Tumor Growth in hMUC-1.Tg Mice Vaccinated with Ad-sig-hIIMUC-1/ecdCD40L Subunit II Vaccine

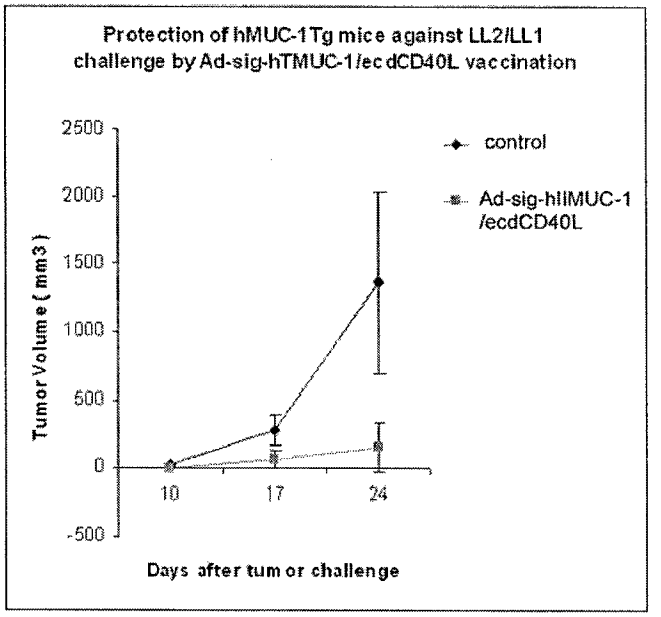


Figure 2C: Prevention Experiment

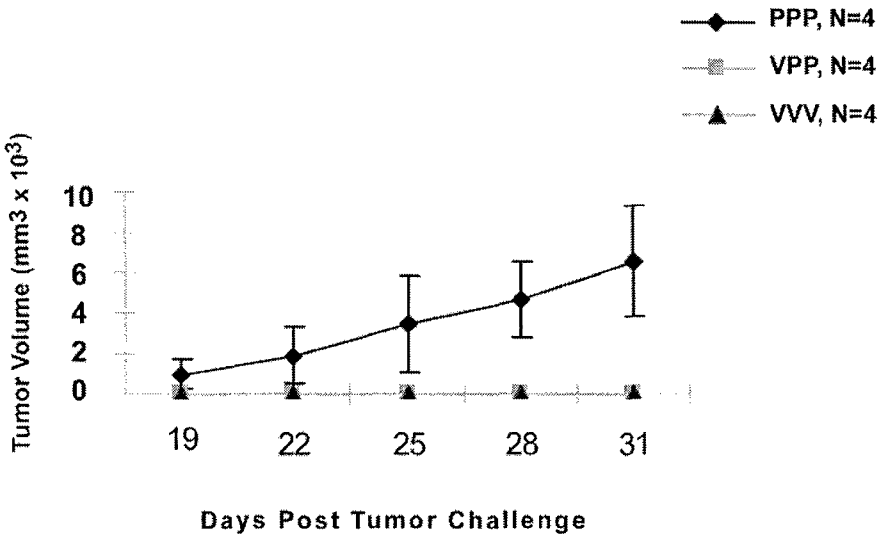


Table I

Vector then Protein Boost

Table I

Testing Group	Week 1	Week 2	Week 3	Week 4
Control	**Vector**	**Vector**	Nothing	Nothing
Treatment 1 (T1)	**Vector**	**Vector**	**Protein**	Nothing
Treatment 2 (T2)	**Vector**	**Vector**	Nothing	**Protein**
Treatment 3 (T3)	**Vector**	**Protein**	Nothing	Nothing
Treatment 4 (T4)	**Vector**	Nothing	**Protein**	Nothing
Treatment 5 (T5)	**Vector**	**Protein**	Nothing	**Protein**
Negative Control	Nothing	Nothing	Nothing	Nothing

Figure 2D

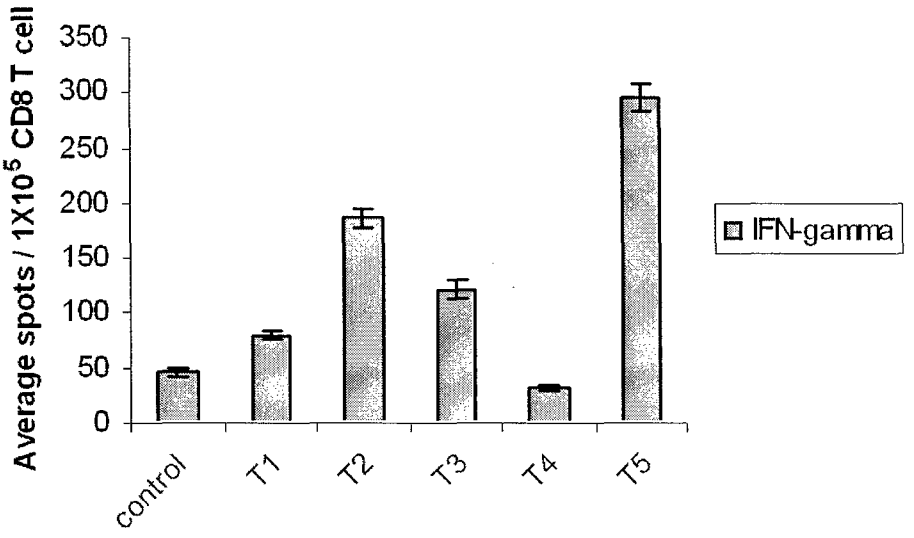
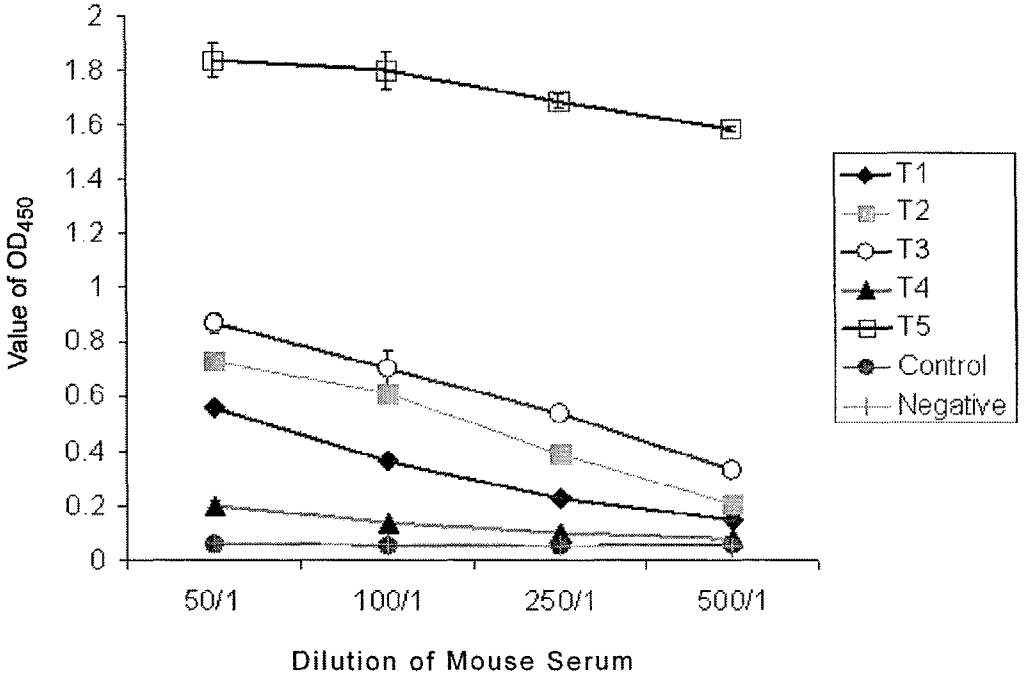


Figure 2E



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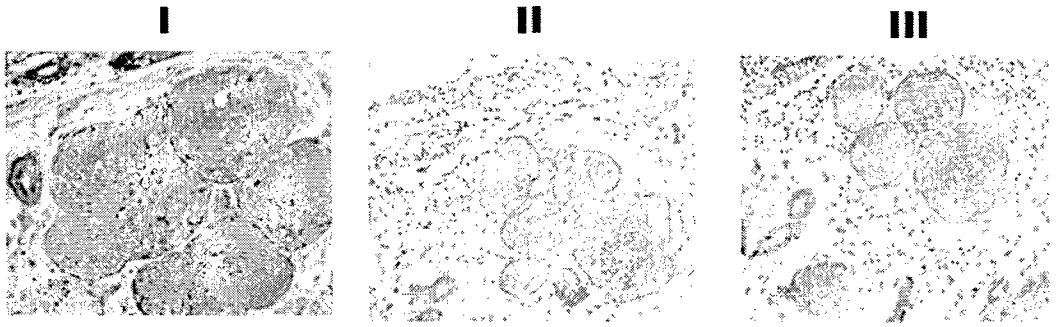


Human Breast Cancer Tissue Stained with Serum from Vaccinated Mice

MUC-1 Peptide Inhibits Staining of Serum From Vaccinated Mice

Human Breast Cancer Tissue was Stained with Serum from Unvaccinated Mice

Figure 2G: Treatment Experiment

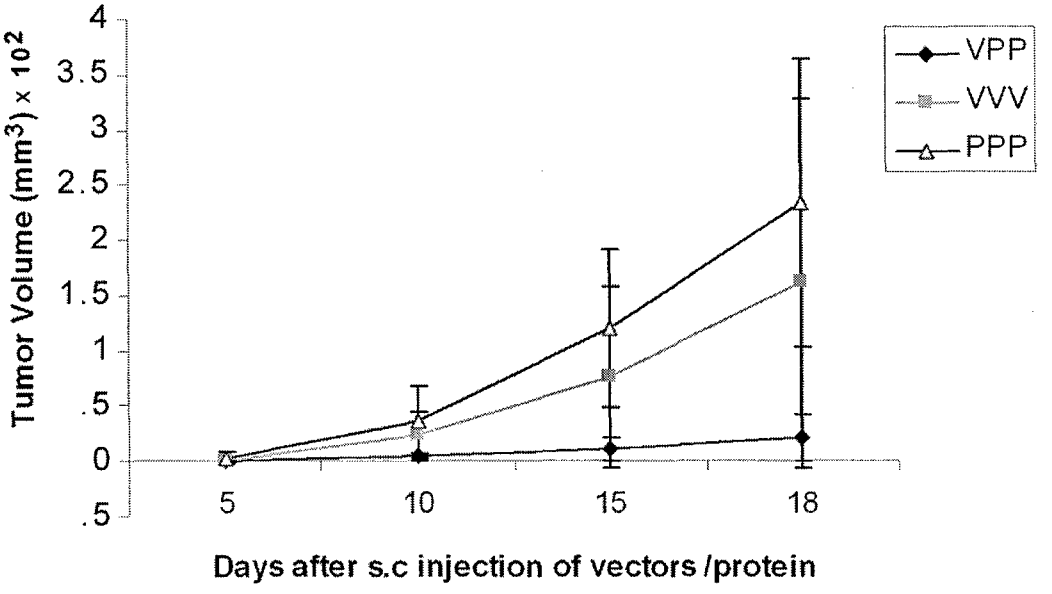


Figure 2H

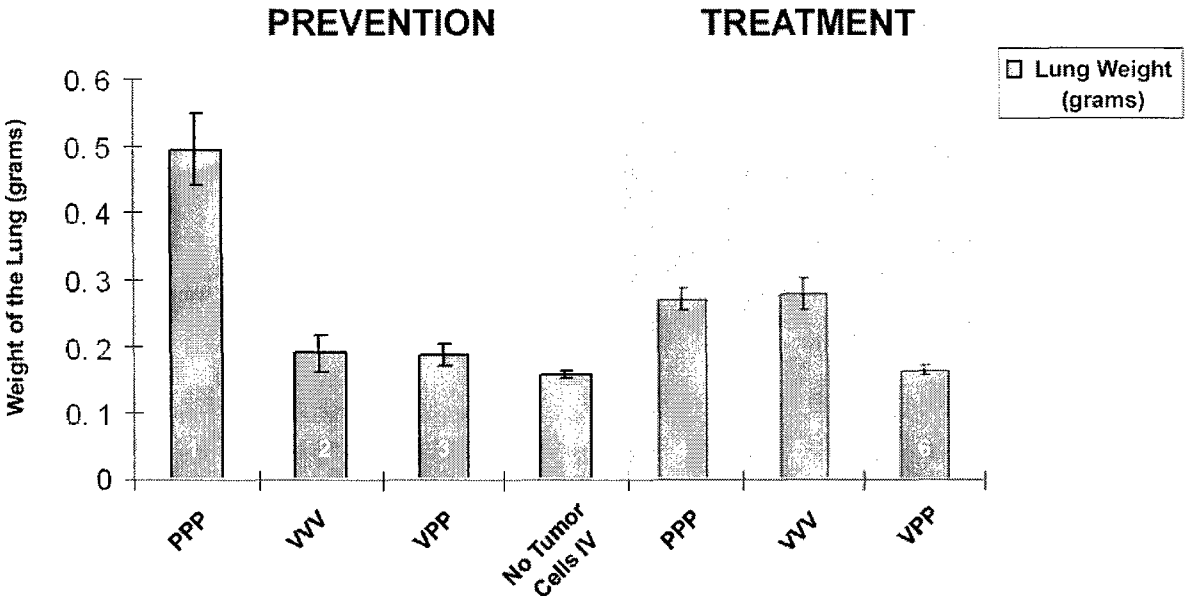


Figure 2I

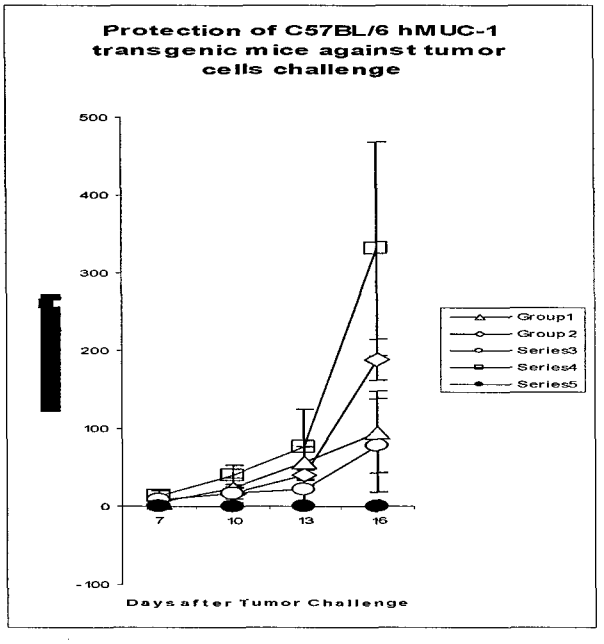


Figure 3A

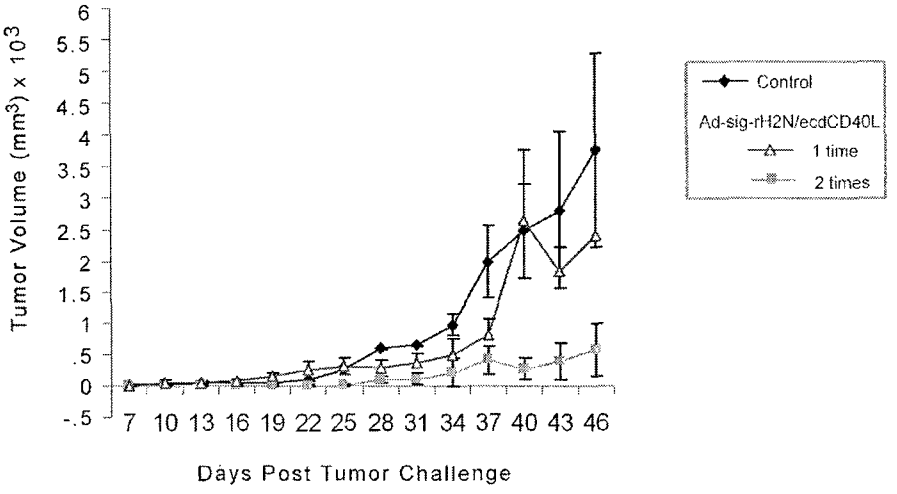


Figure 3B

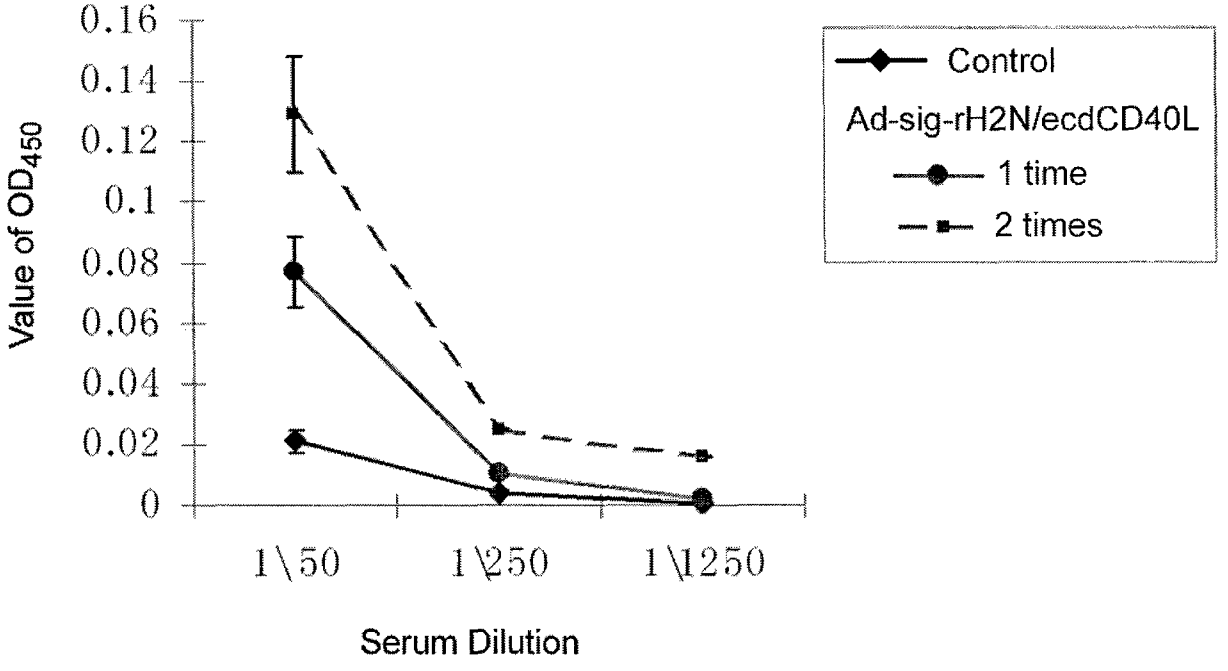


Figure 3C

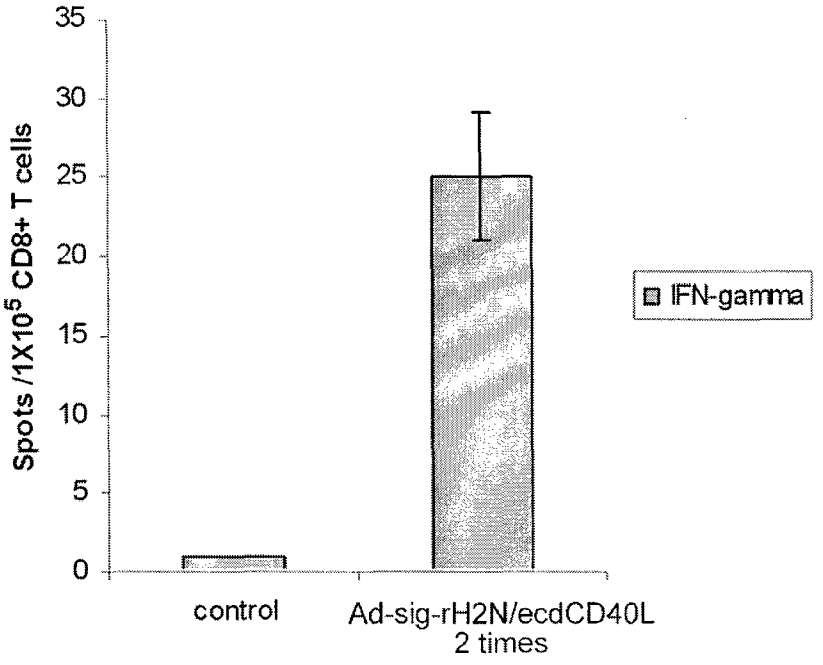
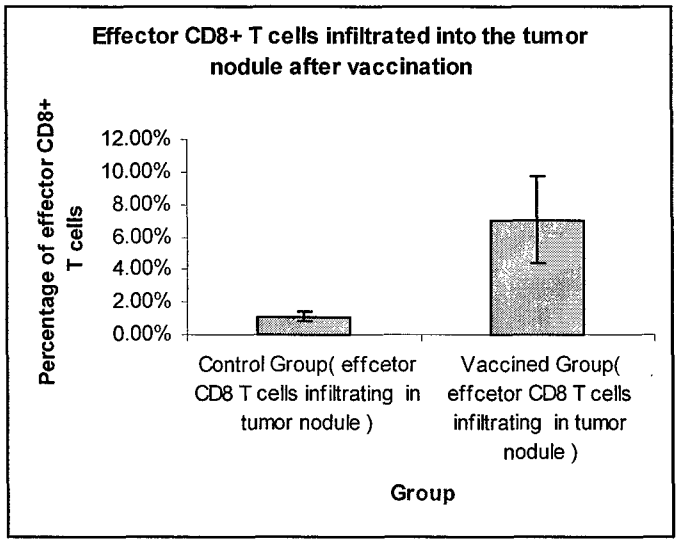


Figure 4



Title Page

Ad-sig-TAA/ecdCD40L Vaccine Overcomes Immune Defect in Old Mice

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Abstract (233)

We have developed an Ad-sig-TAA/ecdCD40L vector anticancer vaccine strategy for the in vivo activation and loading of dendritic cells (DCs). This vector encodes a secretory (sig) transcription unit composed of a tumor associated antigen (TAA) lined to the extracellular domain (ecd) of the CD40 ligand (CD40L). We had shown that the subcutaneous (SC) injection of the vector could break the anergy which existed in TAA.Tg mice for the TAA. This vaccine could confer immunological resistance to the growth of TAA tumor cells for up to one year after vaccination. Since we had shown that this vaccine was CD4 T cell independent, we tested the effect of this vaccine on chronically advanced age mice (18 months). Previous work had shown that these mice exhibited an acquired functional defect in CD4 T cells and quantitative defects in CD4 and C8 T cells. Our results had shown that this vaccine could induce a 10 fold increase in the levels TAA specific CD8 effector T cells in the tumor tissue and a two fold decrease in the levels of FOXP3 negative regulatory CD4 T cells. The vaccine suppressed the in vivo growth of TAA positive T cells and prolonged the survival of 18 month old mice injected SC with the TAA tumor cells. These results suggest that this vaccine strategy can circumvent the acquired and quantitative defects which develop in the immune response during the aging process.

3143 from Intro through Discussion

Introduction (396)

The immune response becomes diminished in age groups in which most epithelial cancers occur due to decreases in the number of naïve CD8 T cells and the acquisition of both quantitative and functional defects in CD4 cells, such as diminished levels of the CD40 ligand (CD40L) on the plasma membrane. Early in life, the immune response becomes tolerant to most of the tumor associated antigens (TAA) because they are present on normal cells from birth. Even when TAA are specifically present in the cancer cells and not present on normal cells, these tumor specific tumor associated antigens are seldom taken up by antigen presenting cells or dendritic cells (DCs) and when this occurs, they are presented on Class II MHC.

Our laboratory has designed an adenoviral vector (Ad-sig-TAA/ecdCD40L) vaccine which is designed to specifically circumvent the functional defects in CD4 helper cells in older individuals and the changes in the cancer host that lead to tolerance. This vector also is suited for the in vivo TAA loading and activation of DCs so as to overcome the above problems in the immune response in cancer patients. The subcutaneous (SC) injection of this vector leads to the release of a fusion protein composed of a TAA linked to the extracellular domain (ecd) of the CD40L. This vaccine has been shown to overcome anergy in TAA.Tg transgenic mouse models, and to induce TAA specific memory cells. This has been accomplished in two TAA.Tg models (rat Her-2-Neu [rH2N] and human Muc-1 [hMUC-1]). We have also shown that it is possible to boost the level of TAA specific T cells and antibodies by SC injections of the TAA/ecdCD40L. The TAA specific antibodies induced by this strategy reacts with human cancer of the breast and prostate.

We were therefore interested in testing whether this strategy could induce an immune response in 18 month old mice so that we could be sure that these animals would response to the vaccine at that late age. Our experiments show that vaccination of 18 month old aged tumor bearing mice increase TAA specific CD8 effector cells and to decrease CD4 FOXP3 negative regulatory T cells in the tumor tissue, and induce complete regressions of existing tumor. The SC injection of the TAA/ecdCD40L protein following the SC administration of

the Ad-sig-TAA/ecdCD40L vector expands the magnitude of the cellular and humoral immune response induced by the vector.

Methods and Materials (1178)

Mice

Six- to 8-wk-old , 3 months ,18 months ,22 months old C57BL/6 mice were purchased from Harlan.

Cell lines

The C57BL/6 syngeneic TC-1 tumor cell line was immortalized with the HPV-16 E6 and E7 genes and transformed with the c-Ha-ras oncogene (25). TC-1 expresses low levels of E6 and E7 and is highly tumorigenic . TC-1 was grown in RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM nonessential amino acids, 1 mM sodium pyruvate, 50 µM 2-mercaptoenthanol, at 37° with 10% CO_2.

Production and purification of recombinant Adenovirus

The E7/ecdCD40L fusion gene was constructed by liganting the aminoterminal end o fhte extracellular domain to an octapeptide linker (NDAQAPKS) to the carboxyl terminal end of a TAA, the amino terminal end of which was linked to a secretory signal sequence.

The strategy for assembling chimeric expression cassettes encoding E7 was as follows: the primer for amplification of E7 was: 5'-TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT GGA G AT ACA CCT AC-3' and 5'-CCG CTC GAG TGG TTT CTG AGA ACA GAT GGG GCA C -3'. This oligonucleotide was cloned into the pcDNA3TOPO vector. And The coding sequences for the extracellular domain mouse CD40 ligand was generated by using the following primers:
5'- GAGAC CTC GAG AAC GAC GCA CAA GCA CCA AAA AGC ATG ATA GAA ACA TAC AGC CAA C-3' and 5'- CCGCGC CCCAAGCTTA TCAGAGTTTGAGTAAGCCAAAAG-3'. The framents were then linked in-frame to the coding sequence of E7 by restriction sites Xba I and XhoI. Then it was directionally cloned into the pShuttleCMV plasmid with the Hind III and Xba I sites. The ecd of the wtCD40L and the full length CD40L were

amplified by PCR primers and cloned into the pShuttleCMV plasmid using the same restriction site Hind III and Xba I sites.

The recombinant adenoviral vectors were generated using the AdEasy vector system . Briefly the resulting plasmid pShuttle E7/ecdCD40L, were linearized by Pme I digestion and then co-transformed into E. coli strain BJ5183 together with pAdEasy-1 . Recombinants are selected with kanamycin and screened by restriction enzyme analysis. The recombinant adenoviral construct is then cleaved with Pac I transfected into 293A cells to produce viral particles. The titer of recombinant adenovirus was decided by the tissue culture infectious dose 50 ($TCID_{50}$) method(27,28).

Purification of recombinant E7/ecdCD40L protein in bacterial system

The E7/ecdCD40L fusion cDNA were inserted into the expression vector p Tri by Xcml and Notl sites. The expression bacterial cell lines Rosetta (DE3) was transfected by pTri E7/ecdCD40L vectors and induced by IPTG for 3 hours at 37°C .The bacterial pallets were harvested and purified by His Selected Nickel Affinity Gel (Sigma)

Flow cytometry analyses of T regulatory cells

To quantify T regulatory cells , the CD4 T cells from lymph node ,spleen and tumor nodule were respectively stained by two different kinds marker , CD4CD25 and CD4FoxP3 with FITC- or PE-conjugated anti-mouse monoclonal antibodies (Pharmingen ,eBiscience) for 30 min on ice, prior to immunostaining with labeled Abs. The T cells were first incubated with a Fc-γ blocking antibody (anti-mouse CD16/CD32 antibody) to avoid the nonspecific binding of mAbs to Fc-γ receptors. The cells were then washed twice, fixed in 4% paraformaldehyde, and analyzed using a Becton Dickinson flow cytometer (FACS Calipur).

Tetramer staining

PE-labeled H-2D^b tetramer containing HPV16 $E7_{49-57}$ peptide(RAHYNIVTF) was purchased from Beckman Coulter and used for the analysis of peptide specific CTL immunity. Ten days after immunization,10^6 erythrocyte-depleted spleen cells were stained by 10ul of tetramer together with 1/100 diluted fluorescein isothiocyanate(FITC)-anti mouseCD8a(clone53-6.7 ,BD Pharmingen) in 100ul PBS supplemented with 3% FCS and incubated at room temperature for 30 minutes, then washed with 3ml of PBS. Then, following centrifugation, the cell pellet was resuspended in 500ul of PBS/0.5% paraformaldehyde for FACS analysis. Tetramer positive and CD8+ cells are shown as a percentage of total spleen cells.

Cytokine Profile by ELISPOT Assays

The presence of E7-specific effector T cells in the immunized mice was also assessed by carrying out ELISPOT assays, as previously described (22, 23). Briefly, splenocytes obtained from mice vaccinated with each of the different vectors were-restimulated in vitro by culture with the TC-1 cell line (responder-to-stimulator ratio=25/1) in the presence of 10 U/ml IL-2 for 48 hours. Re-stimulated splenocytes were then plated in 96-well nitrocellulose filter plates (5 X 10^4 cells in 100 microliters). The wells were pre-coated with rat anti–mouse anti–IFN-antibody or anti–IL-4 antibody. After incubation for 24 hours at 37°C/5% CO_2, the plates were then washed with PBS, and the presence of cytokine-producing spleen cells was detected by incubation at 4°C with biotinylated goat anti–rat secondary antibody, followed by 100 microliter/well of horseradish peroxidase avidin D. To this was added 150 microliter/well freshly prepared substrate buffer (0.4 mg/ml 3-amino-9-ethyl-carbazole in a total of 50 ml 0.05 mol/L sodium acetate buffer) and 20 microliter 30% H_2O_2. The stained spots corresponding to IFN producing cells or to IL-4 producing cells, were enumerated under a dissecting microscope.

Cytotoxicity Assay

Mononuclear cells from the spleens of these mice were incubated with mitomycin C–treated TC-1 cells in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 5 mM 2-mercaptoethanol, 2 mM glutamine, 1mM pyruvate, and nonessential amino acids for 5 days. To perform the cytotoxicity assay, firstly TC-1 tumor cells / (target cells) were labeled with the red-fluorescent dye PKH26 (Sigma, St Louis, MO) according to the manufacturer's specifications. In brief, the target cells were washed in PBS then resuspended at 10^7 cells/ml in solution . PKH26 dye was added to a final concentration of 2 μM, mixed and incubated at room temperature. After 5 min, the reaction was quenched with 3 volumes of FCS and the cells were washed an additional 3 times in RPMI/10% FCS medium then 5 X 10^3 labeled TC-1 cells were incubated with the stimulated splenic mononuclear cells (effector cells) at a different effector/target ratio for 4 hours at 37°C, in culture media containing 5% FBS. At the end of the incubation, mononuclear cell–mediated cytotoxicity was stained for intracellular Caspase-3 according to the manufacturer's protocol(BD PharMingen) ,double positive cells was determined by flow cytometry on live gated $PKH26^+$ cells.

In Vivo Efficacy Experiment in Mouse Model

Mice (5 or 10 per group) were challenged by subcutaneous injection of 5 x 10^5 TC-1 cells were injected subcutaneously ,In the next day , the mice were vaccinated via subcutaneous injection with 1X10^8 PFU Ad-sig-E7/ecdCD40L, One week later, mice were boosted with the same adenoviral vector regimen as the first vaccination or followed by 10ug recombinant E7/ecdCD40L protein every week . Tumor volumes were measured in centimeters by caliper. One month later , the tumor free mice were rechallenged by 1 x 10^7 TC-1 cells

and the volume was calculated as tumor volume= length x (width^2)/2 (this assumes an ellipitical shape.

Statistics

All parameters were analyzed using Student's t test, or ANOVA followed by Scheffé's procedure for multiple comparisons as post-hoc analysis; all data shown is presented as mean ± S.E. of the mean (S.E.).

Results (1018)

Levels of CD8 Effector T Cells in Tumor Tissue Following Ad-sig-TAA/ecdCD40L Vector Vaccination of Young Mice.

We had shown previously that the SC injection of the Ad-sig-TAA/ecdCD40L vector activated and tumor antigen loaded DCs which then migrated to the regional lymph nodes where the DCs resulted in an increase in the levels of the TAA specific CD8 T cells. One question left unresolved by these earlier studies was whether these TAA specific effector CD8 T cells reached the tumor tissue in the extravascular space. In order to test if the levels of CD8 effector T cells in the tumor tissue would be increased following vaccination, we minced the SC tumor nodules of young (2 months old) rH2N.Tg mice before and after two SC injections of the Ad-sig-rH2N/ecdCD40L vector. Single cell suspensions were generated from the tumor tissue after mincing and treatment with 0.03% DNAse I and 0.14% collagenase I, and filtration through Nylon mesh. We found that the percentage of CD8 T cells with the immunophenotype of effector T cells (CD8+, CD44+, LY6C+and CD62L-) was increased in the tumor tissue after vaccination (see Figure 1A). This suggests that the suppression of the growth of the rH2N positive tumor cells in the rH2N.Tg mice following Ad-sig-rH2N/ecdCD40L vaccination is mediated in part by an increase in the trafficking of effector T cells into the tumor tissue.

RNA was isolated from these tumor infiltrating CD8 effector T cells and the pattern of gene expression was compared before and after vaccination using the Affymetrix gene expression system. We also examined the expression level of the 21 known chemokine receptors and ligands in the effector T cells which were infiltrating the tumor tissue. The level of mRNA transcript encoding the CCL3 (2.8 fold increase) and CCR5 (16 fold increase) chemokines were increased in the CD8 effector T cells in the tumor tissue after vaccination. These two chemokines are involved in the targeting of T cells to the extravascular sites of tissue inflammation. The chemokine pathway plays a major role in the trafficking of effector and memory T cells from the lymph nodes draining sites of vaccination or infection to the tissue sites harboring inflammation or infection (42-43).

Levels of Antigen Specific CD8 T cells Before and After Vaccination in Old Mice.

The first test of the Ad-sig-TAA/ecdCD40L strategy was to vaccinate C57Bl/6J mice which were 18 months old with one subcutaneous (SC) injection of the $1x10^8$ IU of Ad-sig-E7/ecdCD40L vector followed in 7 days by one SC injection of the E7/ecdCD40L protein (10 microgram/injection) as a booster. Seven days later, we sacrificed the mice and then measured the level of the E7 specific T cells in the spleen by ELOSPOT assay. As shown in Figure 1B, the levels of interferon-gamma positive T cells in the spleens of the old mice was increased to 120 antigen specific T cells/100,000 CD8 T cells by vaccination. As shown in Figure 1C, when we vaccinated 18 month old tumor bearing mice with one Ad-sig-E7/ecdCD40L vector SC injection followed by 3 SC E7/ecdCD40L protein injections, the level of interferon-gamma positive cells/100,000 splenocytes was increased to 225 in the old mice.

We then measured the increase of the percentage that antigen specific T cells constituted of total CD8 T cells in the tumor tissue before and after vaccination using E7 tetramers. As shown in Figure 1D, the Ad-sig-E7/ecdCD40L vaccine induced the level of antigen specific T cells in the tumor tissue by 10 fold. We also measured the increase of the T cells as a percentage of the total number of cells in the tumor tissue following vaccination in the old mice. As shown in Figure 1E, the increase in the percentage of the T cells increased over 10 fold after the vaccination in the old mice. Finally, the level of E7 specific cytotoxic lymphocytes (CTLs) in the spleen of the 18 (old) and 2 (young) month old mice was studied by in vitro . The results presented in Figure 2A, show impressive increases in the levels of antigen specific CTLs following the vaccine in the old as well as in the young mice. Again, the level of the increase of the CTLs in the 18 month old mice were less than those seen in the 2 month old mice, but the absolute magnitude of the induction induced by the vaccination was impressive in the 18 month aged mice.

Levels of FOXP3 Positive T Negative Regulatory CD4 T Cells Before and After Vaccination.

Increases CD4 FOXP3 negative regulatory T cells have been reported to limit the degree to which vaccines suppress the degree of immune response to vaccination. Decreases in the level of FOXP3 negative regulatory

CD4 T cells have been reported with vaccination (Daniels GA, Sanchez-Perez L, Kottke T, and Vile EG. Expression of CD40L and the use of demethylating agents to enhance vaccine efficacy in melanoma. AACR 46: 1425, 2005). We therefore measured the level of FOXP3 CD4 T cells in the tumor tissue before and after Ad-sig-E7/ecdCD40L vaccination. As shown in Figure 2B, the Ad-sig-E7/ecdCD40L vaccination decreased the percentage that the CD4 FOXP3 negative regulatory T cells constituted of the total CD4 cells in the tumor tissue in old mice by 2 fold.

Study of Tumor Challenge in Aged Mice Vaccinated with the TAA/ecdCD40L Strategy.

As shown in Figure 3A, the Ad-sig-E7/ecdCD40L induced suppression of the E7 positive tumor growth in the 18 month old mice was almost equal to the level of suppression of the tumor growth in 2 month old mice. We then tested the effect of the protein boosts of the induction of the immune response induced by the Ad-sig-E7/ecdCD40L vector. The endpoint of these studies was in vivo suppression of the E7 tumor growth in C57Bl/6J mice, as measured by the percentage of mice which remained tumor free. As shown in Figure 3B, the SC injection of the E7/ecdCD40L booster protein induced regressions of existing tumor and converted several tumor positive old (18 month) mice to tumor negative mice. These data suggested that the E7/ecdCD40L protein boost could induce complete regressions in existing tumor which was progressive in 18 month old mice.

Discussion (546)

Many workers have shown that as mice age, although the total number of T cells stays the same, the ratio of naïve/memory CD8 cells decreases. This may be due to the involution of the thymus gland which is associated with the failure to maintain adequate levels of IL7, and hormonal changes in puberty. This results in a reduction of the repertoire of CD8 T cells available for the immune response. Aged mice will also show oligoclonal expansion of T cells during immunostimulation. In addition, growth of tumor cell lines in mice for greater than 5 days has been reported to be associated with the emergence of anergy to tumor cell antigens.

Previous studies have indicated that the number of interferon-gamma secreting effector CD8 T cells (IGSC) induced by vaccination is decreased in the elderly vs young test subjects after vaccination. In addition, the kinetics of development of the immune response as measured by the peak day of the IGSC level is slower in older animals and in elderly human subjects than in young test subjects (45). Clearly, the Ad-sig-TAA/ecdCD40L vaccine has overcome this problem.

It has been reported that the level of CD154 (CD40L) on CD4 T cells is lower in older mice and test subjects following exposure to vaccination than is the case in younger test subjects (50). The presence of the CD40L on the TAA/ecdCD40L protein serves to replace the need for CD40L on CD4 cells. However, we do not know to what extent the Ad-sig-TAA/ecdCD40L vaccine is also indirectly inducing increases in the level of CD40L on CD4 T cells, thus overcoming the functional defect of these cells in older mice or test subjects.

Previous studies from other labs have shown that the levels of CD4 FOXP3 negative regulatory T cells is higher in the tumor tissue of older mice than is the case in young mice. We have shown that the Ad-sig-E7/ecdCD40L vector vaccine can induce a 2X decrease in the level of the CD4 FOXP3 negative regulatory T cells in 18 month old mice for a foreign antigen. The combination of increased effector CD8 T cells and

diminished levels of CD4 FOXP3 negative regulatory T cells in the tumor tissue induced by the vaccination is undoubtedly responsible for the conversion of 18 month old mice with tumor progression into tumor free mice.

Approximately one half of the patients treated locally with surgery and or radiation therapy will occur after surgery. To deal with this clinical problem, adjuvant therapy (chemotherapy or hormonal therapy) is given after the surgery. In many cases, even the most intense regimens cannot eradicate all of the metastatic disease. The vaccine outlined above for epithelial neoplasm is one candidate for the post surgical adjuvant treatment of epithelial cancer. The advantage of the Ad-sig-TAA/ecdCD40L vaccination is that it has been shown to be expanded and boosted by the simultaneous administration of chemotherapy (40-Akbulut). Thus, this vaccine could be integrated into existing regimens of adjuvant chemotherapy or hormonal therapy.

Most exciting of all is the fact that this vaccine works in old mice. It is possible that the application of this strategy could solve the problems currently being encountered with vaccination of older subjects for protection of these vulnerable individuals against infectious diseases such as influenza and avail flu.

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Figure Legends

Figures

Fig 1A

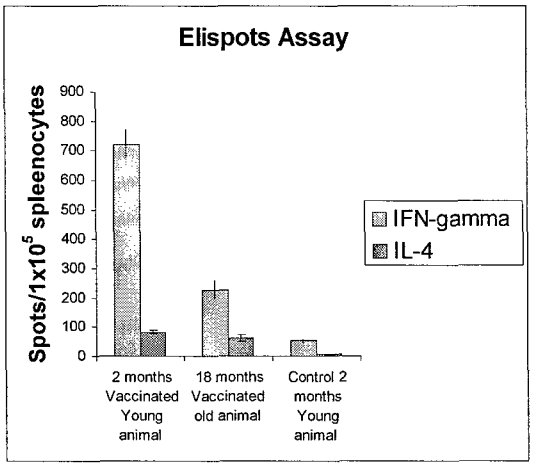


Figure 1B

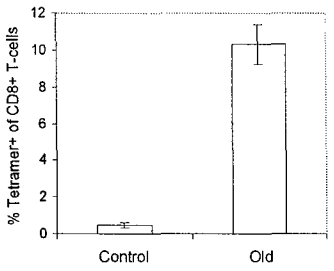
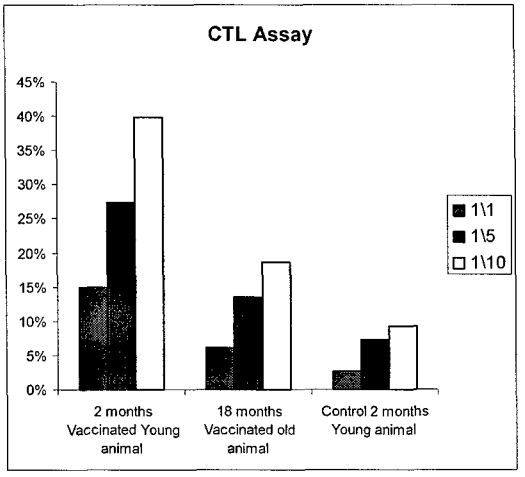


Figure 1C


CTL Assay
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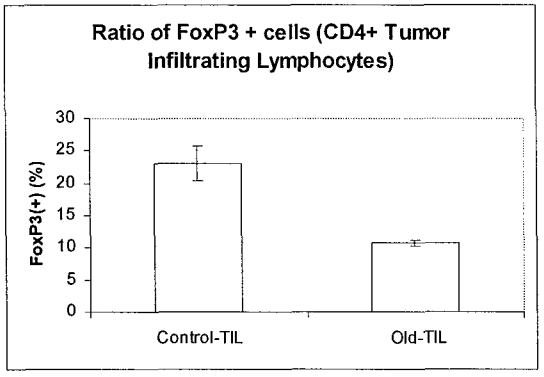
2 months Vaccinated Young animal
18 months Vaccinated old animal
Control 2 months Young animal

Figure 2


Ratio of FoxP3 + cells (CD4+ Tumor Infiltrating Lymphocytes)
FoxP3(+) (%)
30
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Old-TIL

Figure 3A

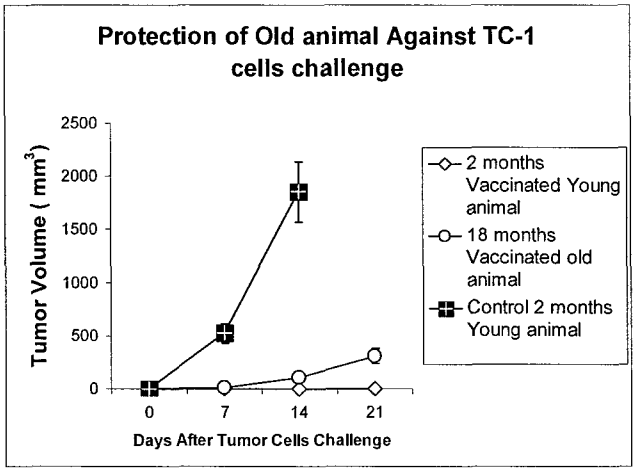
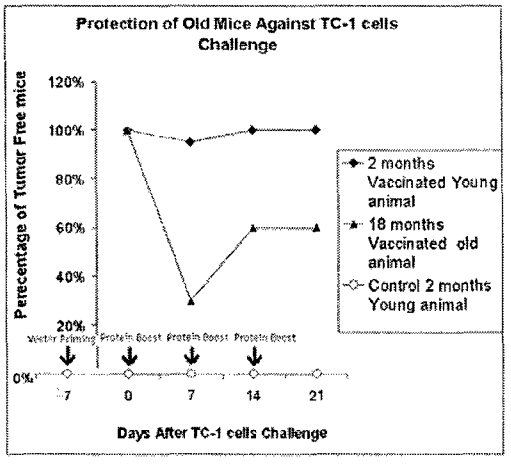


Figure 3B



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Vector Targeting Makes 5-Fluorouracil Chemotherapy Less Toxic and More Effective in Animal Models of Epithelial Neoplasms

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ABSTRACT

Purpose: **5-Fluorouracil (5-FU) has been combined in the past with other drugs for the combination chemotherapy for cancers of the breast, ovary, and colon. These drug regimens were limited by the fact that 5-FU fails to kill nondividing cancer cells at the doses that are safe to deliver. The goal of the present study is to test the feasibility of replacing 5-FU in established 5-FU combination chemotherapy with the Ad-LpCDIRESE1A/5-fluorocytosine (5-FC) system with the purpose of reducing toxicity and increasing efficacy.**

Experimental Design: **We have replaced 5-FU in the weekly combination of CPT-11, folinic acid (FA) and 5-FU (interferon regulatory factor, IRF) chemotherapy by 5-FC and an adenoviral vector that carries the L-plastin (Lp) tumor-specific promoter-driven transcription unit encoding the cytosine deaminase gene linked to the E1A gene by an internal ribosomal entry site element. This combination is called "genetic combination therapy." The goal of using the vector was to decrease the toxicity to normal tissue and to increase the efficacy of therapy in the cancer cells by increasing the concentration of 5-FU sufficiently high that even nondividing cancer cells would be killed by 5-FU through its incorporation into mRNA and consequent inhibition of synthesis of functional proteins. We compared the *in vivo* efficacy of the genetic combination therapy with the conventional IRF combination chemotherapy in a mouse colon cancer model.**

Results: **Both replication-competent and -noncompetent adenoviral vectors carrying L-plastin–driven cytosine deaminase transcription unit when combined with 5-FC, CPT-11, and FA showed increased *in vitro* therapeutic activity that was significantly higher than that of the conventional chemotherapy combination of 5-FU, CPT-11, and FA. Tumor bearing mice treated with the genetic combination therapy showed a statistically significant advantage in terms of increased response rate, response duration, survival, and reduced toxicity when compared with tumor-bearing mice treated with the conventional IRF combination chemotherapy.**

Conclusions: **Replacement of 5-FU– in 5-FU–based combination chemotherapy with the Ad-LpCDIRESE1A vector reduces toxicity and increases efficacy. This is a concept that could be potentially applied widely for many forms of cancer treatment.**

INTRODUCTION

5-Fluorouracil (5-FU) is a component of many chemotherapy regimens that have been used in the past for advanced carcinomas of the breast, ovary, and colon as well as the programs used for the adjuvant therapy for carcinomas of the colon, ovary, and breast (1). One of the limiting factors of a regimen like the CPT-11, 5-FU, and FA is its gastrointestinal toxicity. When 5-FU is given at the maximal doses that are safe to administer systemically, it is usually considered to be toxic only for dividing cells through incorporation of the 5-FU into DNA and binding of 5-FU to thymidylate synthase (1, 2).

The failure of existing 5-FU–based chemotherapy in many advanced cancer patients may be attributable in part to the fact that <10% of neoplastic epithelial cells are proliferating at any given time and therefore most of the cancer cells escape control by 5-FU. If it were possible to safely increase the levels of intravenously administered 5-FU to those at which RNA is sufficiently substituted with 5-FU to suppress protein synthesis (1), then one could kill nondividing cancer cells as well as dividing cancer cells. Unfortunately, the dose increments of systemically administered 5-FU that would be required to prevent protein synthesis and thereby kill nondividing cancer cells would generate unacceptable levels of toxicity to the normal cells of the bone marrow and gastrointestinal tract.

We have therefore proposed to use the L-plastin tumor-specific transcriptional promoter to regulate the expression of the transcription units of an adenoviral vector that is selectively cytolytic to tumor cells, on the basis of the levels of the cytosine deaminase (CD) protein that it produces in vector-infected tumor cells. The L-plastin promoter (3, 4) has been shown to drive the expression of genes in tumor cells but not in normal cells (5–7). The *Escherichia coli* or yeast CD gene (8, 9) catalyzes the

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conversion of the relatively harmless drug, 5-fluorocytosine (5-FC), into the cytotoxic agent, 5-FU. The levels of 5-FU, which are generated by the CD/5-FC system (2) within tumor cells (>300 μmol/L), are much higher than those possible when 5-FU is systemically administered (5 μmol/L).

Adenoviral vectors carrying the CD gene driven by the L-plastin promoter have been shown in our laboratory to sensitize breast, ovarian, and colon cancer cells to the effects of 5-FC (5, 6). We have also placed the gene for E1A, which is necessary for viral replication, downstream of the L-plastin promoter to create a vector that is selectively cytolytic to cancer cells (10). A vector that contains both the CD and E1A genes under control of the L-plastin promoter (AdLpCDIRESE1A) can be used to kill cancer cells through two mechanisms: vector replication within the tumor cells and sensitization of the cancer cell infected by this vector to the effects of 5-FC. The concentration of 5-FU generated in 5-FC–exposed cells infected with the AdLpCDIRESE1A vector are 50 times those possible to safely generate (5 μmol/L) when the 5-FU is administered intravenously and sufficiently high (300 μmol/L) to kill even nondividing cancer cells. This double gene vector has already been shown in our laboratory to have a tumor selective cytotoxic effect that is greater than vectors carrying either the CD or the E1A genes alone (11).

In this report, we have compared the combination of the AdLpCDIRESE1A vector added to CPT-11, 5-FC, and FA chemotherapy (genetic combination therapy) with the conventional combination of CPT-11, 5-FU, and FA. The results of these experiments that are summarized in this report show that "genetic combination therapy" is less toxic and much more effective in suppressing the growth of cancer and extending the survival of mice than is the conventional combination chemotherapy.

MATERIALS AND METHODS

Cells and Reagents. The human cancer cell lines of colon (HTB-38), breast (MCF-7) and prostate (Ln-CaP) were purchased from the American Type Culture Collection (Manassas, VA), and the human epithelial ovarian cancer cell line (Ovcar-5) was obtained from Dr. Thomas C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). All drugs and chemicals were purchased from Sigma (St. Louis, MO). 2′-Deoxyuridine 5′-monophosphate and diammonium salt, [5-^{3}H] (15.0 Ci/mmol) were purchased from Moravek (Brea, CA); IgG-FITC was purchased from eBioScience (San Diego, CA). $\alpha_v\beta_5$ and $\alpha_v\beta_3$ monoclonal antibodies were purchased from Covance (Richmond, CA). Mouse anti-coxsackie-adenoviral receptor (anti-CAR) antibody was kindly provided by Dr. Robert W. Finberg (University of Massachusetts Medical School). Wild type Adenovirus type 5 (Ad5WT) was purchased from the American Type Culture Collection. The construction of the following vectors, AdLpE1A, AdCMVE1A, AdLpCD, AdLpCDIRESE1A, and AdCMVCDIRESE1A, has been described in previous publications from our laboratory (5–7, 11, 12).

Carboxylesterase and Thymidylate Synthase Activities of Tumor Cells. Carboxylesterase (CE) activity of all cell lines was assessed by measuring the hydrolytic conversion of paranitrophenolic acid to paranitrophenol that is catalyzed by CE (13). Thymidylate synthase activity of tumor cells was measured according a tritium-release assay as described previously (14). The incubations were done in quadruplicate.

Immunofluorescent Analysis. Using an antimouse IgG-FITC antibody, we measured the level of expression of CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrin receptors on tumor cells by flow cytometry as described in previous publications (6, 7, 11). To show the *in vivo* tumor specificity of Lp-driven vector normal hepatic tissue and subcutaneous tumor nodules caused by inoculating HTB-38 cells infected with AdLpCDIRESE1A or AdCMVCDIRESE1A vectors by percutaneous injection. We also studied the expression of E1A protein by immunofluorescent staining (Vector M.O.M. Immunodetection kit, Vector Laboratories, Burlingame, CA) using adenovirus type 5E1A antibody from NeoMarkers (Fremont, CA).

***In vitro* Studies of Vector-Infected Cell Lines.** The tumor cells were seeded at a density of 100,000 cells/well in 6-well plates. The attached cells were infected with the several vectors. The first is the AdLpE1A conditionally replication-competent vector with the tumor-specific L-plastin promoter regulating the viral E1A transcription unit (7). The second is the AdLpCDIRESE1A conditionally replication-competent vector carrying a bicistronic transcription unit composed of the cytosine deaminase chemotherapy sensitization gene linked by an internal ribosomal entry site (IRES) to the viral E1A replication gene under the control of the tumor-specific L-plastin promoter (11). The third is the AdCMVE1A replication-competent vector carrying the viral E1A replication gene governed by the cytomegalovirus (CMV) tumor nonspecific transcriptional promoter (7). The fourth is the AdCMVCDIRESE1A replication-competent vector carrying the CDIRESE1A bicistronic transcription unit regulated by the CMV promoter (11). The fifth is the wild-type replication-competent adenovirus (AdWT).

After 2 days of incubation of the infected cells, the supernatant medium of the wells was discarded, and SDS sample lysis buffer (including DTT) was added to the cells. The cells were processed as outlined in a previous publication (11). The steady-state levels of 5-FU generated in the medium from AdLpCD-infected tumor cells were measured.

Effect of Vectors on the Drug Concentration Needed for Half-Maximal Growth Inhibition (IC_{50}) of 5-Fluorouracil, CPT-11, and SN-38. We plated 1–5 × 10^4 cells/well in 96-well plates in 100 μL of culture medium. After an overnight incubation, fresh medium supplemented with various amounts of the test drug were added: for 5-FU, from 0.05 to 410 μmol/L of 14 different concentrations; for CPT-11, from 5 nmol/L to 1.3 mmol/L of 10 different concentrations; and for SN-38, from 10 pmol/L to 10 μmol/L of 7 different concentrations. After a 72-hour incubation period with drug, the medium was exchanged for fresh medium without drug. The next day, 10 μL of MTT reagent as provided in the commercial kit (American Type Culture Collection) were added to each well containing cells. After an incubation period of 24 hours, which was continued until a purple precipitate was visible at 37°C, the plates were then incubated further overnight at room temperature. The results were expressed as the average percentage (in quadruplicate) of the population of cancer cells present before treatment that were left surviving at any time point.

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Animal Model 1. HTB-38 colon cancer cells (3×10^6) were injected subcutaneously into female nude/nude mice (4–6 weeks of age). We injected 1×10^8 plaque-forming units of the AdLpCDIRESE1A vector or the AdLpCD vector intratumorally into subcutaneous nodules (50 mm^3) of the HTB-38 colon cancer cells that developed from the subcutaneous injection of tumor cells in female *nu/nu* mice (Fig. 1). The vector or PBS control injections into the tumor nodules were repeated on days 1, 8, 15, and 22. All of the drugs were given to test mice at doses that were equivalent to human doses: 500 mg/kg/day 5-FC intraperitoneally for 10 days; 150 mg/kg 5-FU intravenously on days 1, 8, 15, and 22; 6 mg/kg/day FA intravenously daily starting on the day of intratumoral vector injection for the vector groups and on days 1, 8, 15, and 22 for the CPT-11 + 5-FU + FA group; and 40 mg/kg CPT-11 intravenously on days 1, 8, 15, and 22 (15). A total of two complete cycles of therapy (each 6 weeks long) were given to all animals. Please see Table 1 in which the treatment groups are summarized. Tumor volumes were measured every 2 days (6, 7).

Data are represented as the mean change in tumor size relative to the tumor size at the beginning of treatment of each animal. Animals were evaluated for toxicities of the treatment regimens every day. The following parameters for toxicity were measured daily: activity, skin color, hunching, and fur status, and the cage bedding was inspected daily for residues of diarrhea. The weight of mice was measured three times a week. A separate set of experiments was carried out in which the vector was injected twice a week (instead of the once a week described above), and the tumor dose injected was 2×10^5 cells instead of 3×10^6 cells.

Animal Model 2. To test the efficacy of the vector in a way that was not limited by diffusion and spread of the vector from the intratumoral injection sites of the vector particles to each of the tumor cells in the tumor nodule, we first incubated the HTB-38 cells *in vitro* at a multiplicity of infection of 30 (100% of the HTB-38 cells were shown previously to be infected at a multiplicity of infection 30) with either the AdLpCDIRESE1A vector or AdWT for 60 minutes. After subcutaneous injection of the vector-infected tumor cells (5×10^5 cells/mouse), we administered the drugs at the same doses used in animal experiment 1 to the assigned groups (Table 1). Two cycles of therapy were given in all groups except group 1. In group 1, intraperitoneal 5-FC injections were given only in the 1st and 2nd week because no tumor appeared. Please see Table 1 in which the treatment groups are summarized.

Table 1 Animal models 1 and 2

Group	Dose
Model 1	
1	AdLpCDIRESE1A + 5-FC + FA + CPT-11 ('genetic combination therapy')
2	AdLpCD + 5-FC + FA + CPT-11 (genetic combination therapy minus vector replication competency)
3	AdLpCDIRESE1A + 5-FC (genetic combination therapy minus FA and CPT-11)
4	AdLpCDIRESE1A + 5-FC + FA (genetic combination therapy minus CPT-11)
5	AdLpCD + 5-FC + FA (genetic combination therapy minus vector replication competency and CPT-11)
6	CPT-11 + 5-FU + FA (standard combination chemotherapy regimen)
7	AdLpCDIRESE1A + FA + CPT-11 (genetic combination therapy minus 5-FC)
8	Control (untreated)
Model 2	
1	*In vitro* AdLpCDIRESE1A and *in vivo* chemotherapy: ip 5-FC + iv FA + iv CPT-11 (the genetic combination therapy)
2	*In vitro* AdLpCDIRESE1A and *in vivo* chemotherapy: iv FA + iv CPT-11 (genetic combination therapy minus 5-FC)
3	*In vitro* Ad-LpCD vector and *in vivo* chemotherapy: ip 5-FC + iv FA + iv CPT-11 (genetic combination therapy minus vector replication competency)
4	*In vitro* AdWT and *in vivo* chemotherapy: iv 5-FU + iv FA + iv CPT-11 (iv)
5	*In vitro* PBS and *in vivo* chemotherapy: iv 5-FU + iv FA + iv CPT-11 (iv)

Abbreviations: iv, intravenous; ip, intraperitoneal.

Statistical Analysis. IC_{50} values were calculated according to the median effect principle. The differences among the results of the various groups were compared by the Student's *t*

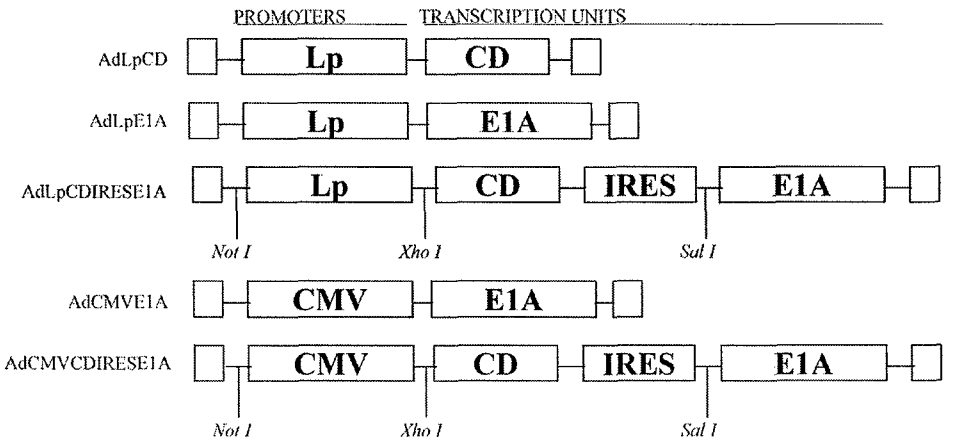

Fig. 1 The maps of the therapeutic transcription units in adenoviral vectors. E1A, adenoviral replication gene; Lp, L-plastin.

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AQ: E test. One-way ANOVA (with LSD post hoc comparisons) and Mann-Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analyses were done according to the Kaplan-Meier method, and the log-rank test was used for survival comparisons.

RESULTS

In vitro Studies in Cell Lines

To evaluate the reasons for differences in response to the CPT-11, 5-FU, and FA chemotherapy and the genetic combination therapy (the AdLpCDIRESE1A vector, CPT-11, 5-FC, and FA) in the cell lines and animal models, we studied the CE and thymidylate synthase activity of the human tumor cell lines. The CE activity of Ln-CaP (human prostate cancer), MCF-7 (human breast cancer), and Ovcar-5 (human ovarian cancer) cells were similar. However, the HTB-38 human colon carcinoma cells had six times more CE activity than the other cell lines, which is consistent with the finding that this cell line converts more CPT-11 into SN-38 (Table 2). The thymidylate synthase activity (14), which was expressed as the amount of ^3H_2O (fmol) formed in 1 minute/mg of protein, was lowest in the HTB-38 colon cancer cell line and highest in the Ovcar-5 ovarian cancer cell line (Table 2).

T2

To characterize the cell lines with respect to differences which might alter the infectibility of the target cell lines, we studied the expression of CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors on tumor cells (16). The percentage of $\alpha_v\beta_3$, $\alpha_v\beta_5$ integrin receptor, as well as the CAR-positive cells was measured by flow cytometry. The percentage of cells positive for CAR and $\alpha_v\beta_5$ integrin receptors varied among the tumor cell lines. More than half of the tumor cell lines have significant percentages of CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrin receptor-positive cells (Table 3). According to these results, Ln-CaP seems to be the most sensitive cell line in terms of taking up the adenoviral vectors. We have previously shown that the E1A gene expression of the human tumor cell lines infected by Lp-driven vectors carried the E1A transcription unit (7). The results suggest that the HTB-38 cell line should be infectable by the adenoviral vectors as well.

T3

AQ: F

Specificity of Expression of the L-plastin Promoter Driven Vector Transcription Units

We then used Western blot analysis (Fig. 2) to study the E1A expression after exposure of cell lines to various vectors and 2 days of incubation of all vector-infected tumor cell lines. The results of these experiments showed bands specific for E1A polypeptides (35–46 kDa). No protein bands are visible from the control cells that were not exposed to E1A-containing vectors.

F2

AQ: G

Table 3 The percentage of tumor cells positive for the CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ receptors as measured by fluorescence-activated cell sorter analysis

Cell lines	CAR (%)	AvB5 (%)	AvB3 (%)
Ln-CaP	82.0 ± 13.8	64.1 ± 0.8	39.3 ± 1.6
MCF-7	37.5 ± 7.5	94.7 ± 3.8	19.1 ± 0.8
Ovcar-5	63.6 ± 19.6	88.2 ± 4.6	46.8 ± 1.4
HTB-38	77.9 ± 17.6	65.6 ± 5.8	29.5 ± 2.2

Abbreviations: AvB5, $\alpha_v\beta_5$; AvB3, $\alpha_v\beta_3$.

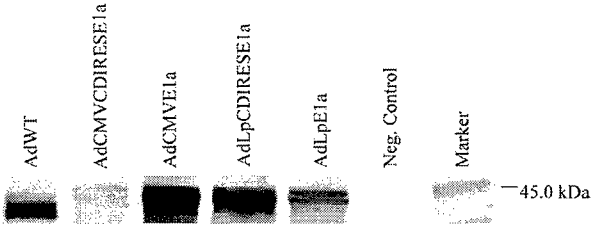

Fig. 2 Western blotting of E1A polypeptides produced in vector-infected HTB-38 cells. The E1A region encodes a series of related proteins (35 to 46kDa).

To test whether the L-plastin–driven bicistronic CDIRESE1A transcription unit was expressed in a tumor-specific manner in the AdLpCDIRESE1A vector-infected cells (5–7), we injected percutaneously either the AdLpCDIRESE1A or the AdCMVCDIRESE1A vectors into normal liver (Fig. 3*C* and *D*) or subcutaneous tumor nodules (Fig. 3*A* and *B*) and stained histologic sections of the injected tissue for E1A expression (*green color*) The tumor nodules were positive for E1A whether injected by the AdLpCDIRESE1A or AdCMVCDIRESE1A vectors (Fig. 3*A* and *B*, respectively) whereas the normal liver tissue was positive for E1A after injection with the AdCMVCDIRESE1A (Fig. 3*D*) but negative for E1A after injection with the AdLpCDIRESE1A vector (Fig. 3*C*). These results show that the expression of the transgenes in the AdLpCDIRESE1A vector-infected cells is tumor specific.

F3

Effect of the AdLpCDIRESE1A Vector on IC_{50} Values of CPT-11, SN-38, and 5-FU

The steady-state levels of 5-FU generated in the medium from AdLpCD-infected MCF-7 cells exposed to 5-FC were >340 μmol/L. These levels are far in excess of the IC_{50} of the MCF-7 breast cancer cells (Table 4) and far above the 5 μmol/L levels that are generated by systemic administration of 5-FU.

T4

Table 2 CE and TS activity of the tumor cell lines and the IC_{50} values of the 5-FU, CPT-11, and SN-38 in these cell lines.

Tumor cells	TS activity *	5-FU_{IC50} (μmol/L)	CE activity †	$CPT11_{IC50}$ (μmol/L)	$SN\text{-}38_{IC50}$ (μmol/L)	$CPT11_{IC50}/SN\text{-}38_{IC50}$
Ln-CaP	6813	0.1	0.3	0.9	4.2×10^{-5}	2.1×10^{4}
MCF-7	5441	0.7	0.3	14.2.	5×10^{-4}	2.8×10^{4}
Ovcar-5	7954	2.8	0.3	21.4	4.6×10^{-1}	4.7×10^{1}
HTB-38	4342	1.6	1.99	5.8	5.1×10^{-5}	1.1×10^{5}

* fmol/minute/mg protein.
† mU/mg protein.

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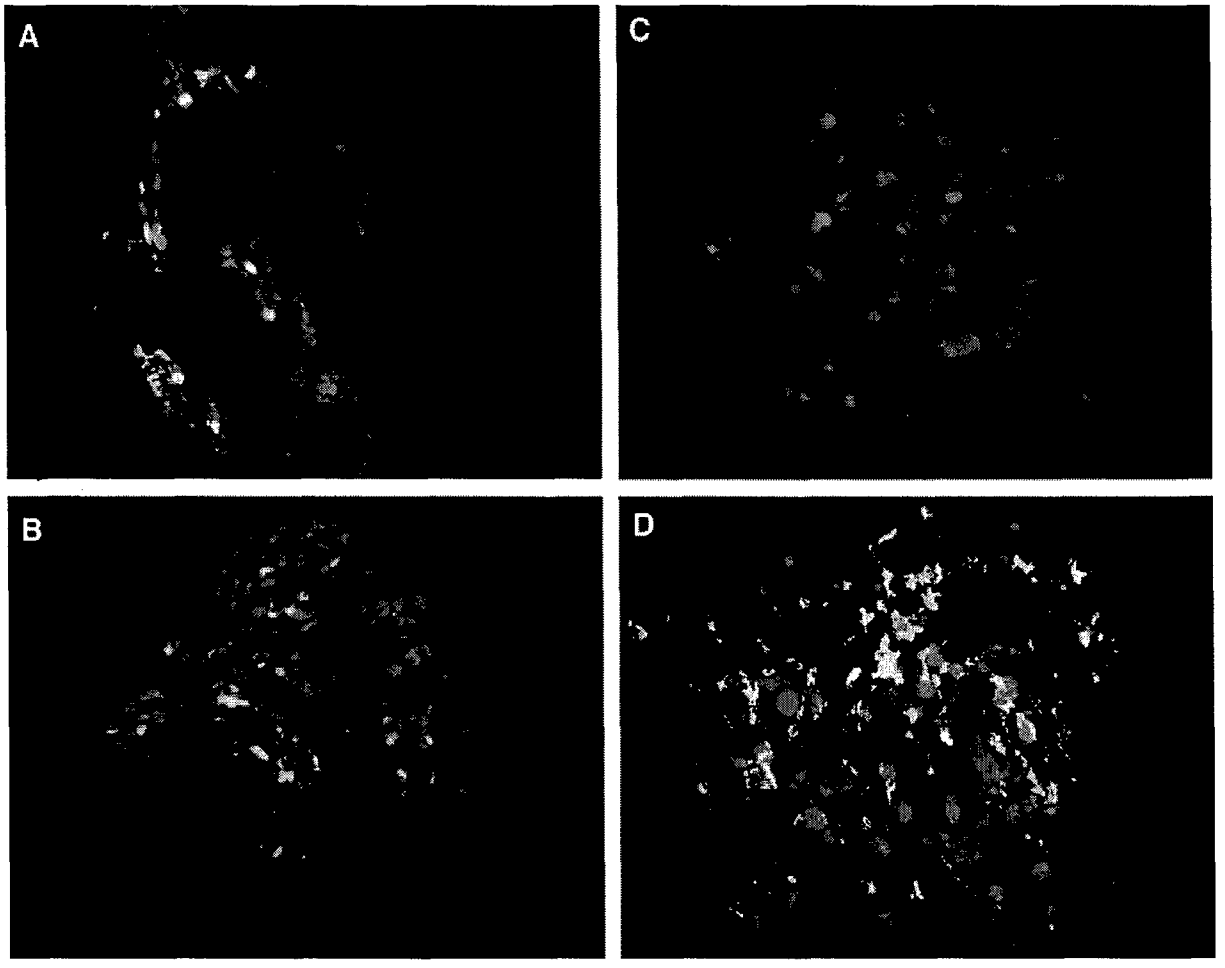

Fig. 3 Tumor-specific expression of the E1A gene following injection with vectors carrying the CMV or L-plastin promoters. In this study, *green color* indicates expression of E1A peptides. *A*, in the tumor nodule injected with the AdLpCDIRESE1A vector, the green fluorescence is detectable showing that the E1A polypeptides (which are stained green) are produced by the L-plastin–driven E1A gene and are present with the nuclei of the tumor cells that are stained *red*. *B*, the tumor nodule that was injected with the AdCMVCDIRESE1A vector also showed expression of the E1A polypeptides. *C*, in the normal liver injected with the AdLpCDIRESE1A vector, there is no green fluorescence of E1A polypeptides. *D*, the liver was injected with the AdCMVCDIRESE1A vector. The nuclei of the liver cells are stained red whereas the E1A polypeptides are stained green in the cells injected with the vector carrying the tumor nonselective CMV promoter. These data show that the normal cells can be infected by the vector and can express E1A peptides in the presence of the CMV promoter.

These levels are similar to those reported previously to result in the inhibition of protein synthesis (1) and thereby to the death of nondividing cancer cells.

We then tested whether the IC_{50} of 5-FU, CPT-11, and SN-38 was decreased by exposure of the test cells to the AdLpCDIRESE1A vector. When the AdLpCD vector plus 5-FC and FA were added to the test cells at a multiplicity of infection of 10, the IC_{50} value of CPT-11 decreased 65 to 2,200 times

Table 4 The IC_{50} values of CPT-11 and SN-38 in tumor cells after exposure to the AdLpCD or AdLpCDIRESE1A vectors at a multiplicity of infection of 10.

	CPT-11_{IC50} (μmol/L)		SN-38_{IC50} (μmol/L)	
Tumor cells	AdLpCD	AdLpCD-IRESE1A	AdLpCD	AdLpCD-IRESE1A
Ln-CaP	$3.5 \pm 1.4\times10^{-3}$	$2.5 \pm 0.9\times10^{-4}$	$6.8 \pm 5.14\times10^{-12}$	$3.5 \pm 1.2\times10^{-12}$
MCF-7	$6.2 \pm 2.3\times10^{-3}$	$1.4 \pm 1.5\times10^{-3}$	$2.8 \pm 1.8 \times 10^{-8}$	$3.9 \pm 1.6\times10^{-10}$
Ovcar-5	$2.8 \pm 1.1\times10^{-2}$	$8.2 \pm 4.4\times10^{-3}$	$6.1 \pm 3.5 \times 10^{-8}$	$5.1 \pm 1.8\times 10^{-8}$
HTB-38	$8.8 \pm 1.7\times10^{-3}$	$4.3 \pm 2.3\times10^{-3}$	$7.8 \pm 4.2 \times10^{-12}$	$8.2 \pm 1.3\times10^{-12}$

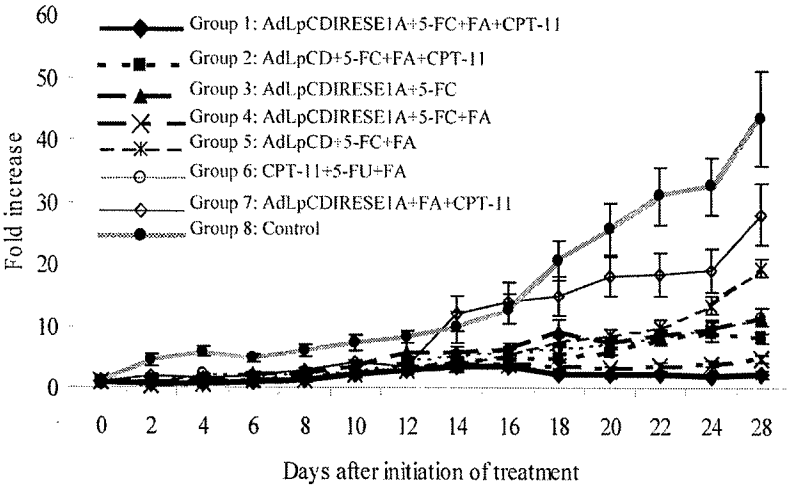

Fig. 4 Tumor response of colon cancer in mice after treatment in animal model 1. The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with intraperitoneal 5-FC daily for 10 days and 1 day of intravenous FA and CPT-11 (genetic combination therapy, group 1) on the growth of HTB-38 cells is greater than that of the conventional CPT-11, 5-FU, and FA combination chemotherapy (group 6) and of other treatment groups ($P < 0.05$).

(Table 4). When the AdLpCDIRESE1A replication-competent vector plus 5-FC and FA was added to the cells, the decrease in the CPT-11 IC_{50} was 5 to 20 times the decrease seen with the AdLpCD replication-deficient vector. The decrease in the CPT-11 IC_{50} with both AdLpCD and AdLpCDIRESE1A plus 5-FC and FA was significantly higher when compared with 5-FU and FA ($P < 0.05$; data not shown). In contrast, when the AdLpCDIRESE1A vector plus FA without 5-FC was combined with CPT-11, the decrease of the IC_{50} value of CPT-11 was between 7- and 200-fold (data not shown). These studies show that the *in vitro* activity of CPT-11 is potentiated by the addition of the AdLpCDIRESE1A vector. Moreover, this vector sensitization does not depend on the replication competency of the vector but on the presence of the CD protein and 5-FC.

Replacement of 5-FU in the Conventional CPT-11, 5-FU, and FA Combination Chemotherapy by Intratumoral Injection of the AdLpCDIRESE1A Vector and *In vivo* 5-FC (Genetic Combination Therapy), Animal Model 1

The 5-FU–based chemotherapy is the choice for the treatment of colorectal cancer in humans. Therefore, we used the HTB-38 cells for the *in vivo* studies of the genetic combination therapy. Test mice were given the treatments outlined in Table 1.

Response Studies. The growth of the HTB-38 colon cancer cell line in nude mice was suppressed more by intratumoral injection of the AdLpCDIRESE1A vector given in concert with intraperitoneal 5-FC, intravenous FA, and intravenous CPT-11 chemotherapy (group 1) than it was by conventional CPT-11, 5-FU, and FA combination chemotherapy (group 6) as shown in Fig. 4. The duration of tumor response among the animals treated with the genetic combination therapy (AdLpCDIRESE1A/5-FC/FA/CPT-11, group 1) was statistically significantly longer than the duration of the response among animals treated with regimens not containing the AdLpCDIRESE1A/5-FC combination ($P < 0.001$).

Survival Studies. The mice treated with AdLpCDIRESE-1A/5-FC/FA/CPT-11 (the genetic combination therapy or group 1) lived much longer than did the mice treated with conventional interferon regulatory factor combination chemotherapy (group 6) or the other control groups ($P < 0.01$, Fig. 5). We then tested for the effect of increasing the frequency of the AdLpCDIRESE1A vector injections from once a week to twice a week. A survival advantage was seen in this latter model, but this advantage was lost by 12 weeks (data not shown). This suggested that additional cycles of therapy might be one way to increase the success of the outcome.

Toxicity Studies. As shown in Table 5, the mice given the conventional CTP-11, 5-FU, and FA combination chemotherapy had statistically significantly more diarrhea than that observed in mice treated with the genetic combination therapy, which involves the combination of AdLpIRESE1A/5-FC/FA/CPT-11 (32.5 *versus* 2.5%, respectively, $P = 0.001$).

Response of the HTB-38 Colon Cancer Cells to *In vitro* Infection with the AdLpCDIRESE1A Vector Infection and *In vivo* CPT-11, 5-FC, and FA Chemotherapy (Animal Model 2)

To test if we could improve the outcome of the genetic combination therapy, we infected the HTB-38 cells with AdLpCDIRESE1A vector *in vitro* under conditions that would result in infection of 100% of the HTB-38 cancer cells before the injection of the tumor cells into the subcutaneous space of the test animals. After subcutaneous injection of HTB-38 tumor cells that had been infected *in vitro* with either the AdLpCDIRESE1A vector, the AdLpCD vector, or the AdWT virus, we treated the mice with the programs outlined in Table 1.

Response Studies. None of the mice treated with the genetic combination therapy (group 1) exhibited regrowth of the tumor cells at the injected sites during the 5 months of follow-up whereas the other treatment groups showed regrowth of tumor after chemotherapy (Fig. 6). In each of the groups in which the colon cancer cell line HTB-38 was infected *in vitro* with a replication-competent vector (groups 1 and 4) and in which *in vivo* 5-FC (in the case of the AdLpCDIRESE1A) or 5-FU (in the case of AdWT) was given, there was a statistically significant reduction in the tumor growth rates ($P < 0.05$, Fig. 6).

Survival Studies. There was a survival advantage of the genetic combination therapy (group 1) as compared with the use of *in vivo* administration of the conventional CPT-11, 5-FU, and

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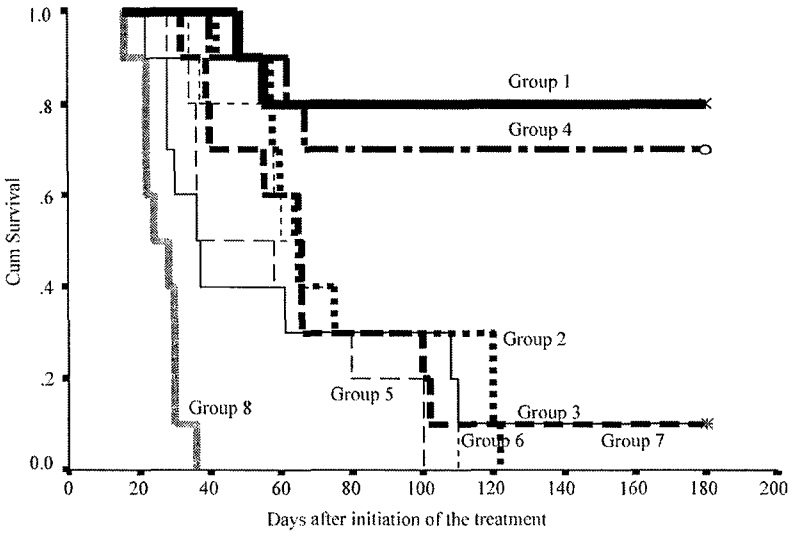

Fig. 5 Survival of mice after treatment in animal model 1. The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and one day of intravenous FA and CPT-11 (genetic combination therapy, group 1) on prolongation of survival of mice carrying subcutaneous nodules of HTB-38 cells is greater than that of the conventional CPT-11, 5-FU, and FA combination chemotherapy, group 6 ($P < 0.01$).

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Table 5 Common toxicities seen in the treatment groups of animal model 1 (percentage of the total cycles in the first 4 weeks of treatment)

Groups	Treatment	Diarrhea	Weight changes †
1	AdLpCDIRESE1A + 5-FC + FA + CPT-11	2.5	10.4 ± 1.0
2	AdLpCD + 5-FC + FA + CPT-11	0	3 ± 0.6
3	AdLpCDIRESE1A + 5-FC	0	8.5 ± 0.6
4	AdLpCDIRESE1A + 5-FC + FA	0	4.1 ± 0.5
5	AdLpCD + 5-FC + FA	0	6.7 ± 0.6
6	CPT-11 + 5-FU + FA	32.5 *	3 ± 0.6
7	AdLpCDIRESE1A + FA + CPT-11	2.5	4.8 ± 0.7
8	Control	0	8.6 ± 0.6

* The mice in the chemotherapy regimen (CPT-11 + 5-FU + FA) or group 6 had significantly more episodes of diarrhea (grade 1 or more) than the other groups ($P = 0.001$).

AQ: I

† Denotes the percentage of change of weight during the 1st cycle of the treatment.

FA combination chemotherapy (group 5) as shown in Fig. 7. When 5-FC was deleted from the genetic combination therapy, the survival advantage of the genetic combination therapy over the conventional CPT-11, 5-FU, and FA combination chemotherapy was lost (*e.g.*, group 3 in Fig. 7). These results indicated that the outcome of therapy depended on the conversion of 5-FC to 5-FU within the tumor cells.

DISCUSSION

We have tested whether it is possible to increase the efficacy and decrease the toxicity of 5-FU–based combination chemotherapy for advanced cancer by using a vector to target 5-FU therapy to tumor cells and spare the normal cells of the body. The combination of the AdLpCDIRESE1A conditionally replication-competent adenoviral vector with CPT-11, 5-FC, and FA chemotherapy, which is called the genetic combination therapy, is statistically significantly superior to the conventional CPT-11, 5-FU, and FA combination chemotherapy with respect to tumor response and survival. These effects may be attributable to the high levels of 5-FU gener-

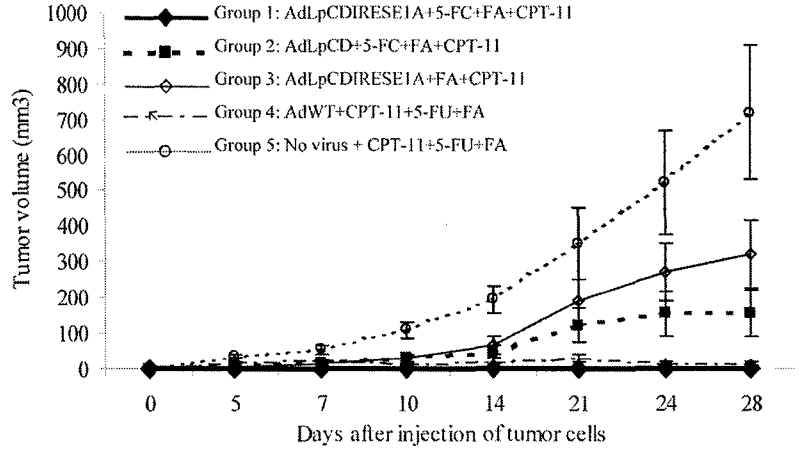

Fig. 6 Tumor response of colon cancer in mice after treatment in animal model 2. The effect of *in vitro* infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and 1 day of intravenous FA and CPT-11 (genetic combination therapy, group 1) on tumor growth is greater than that of the conventional CPT-11, 5-FU, and FA combination chemotherapy, group 5 ($P < 0.05$).

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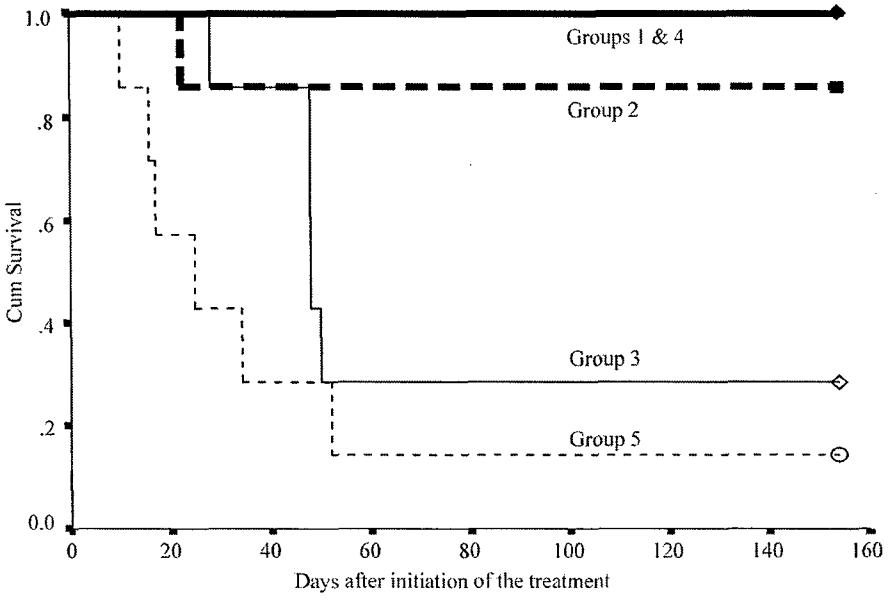

Fig. 7 Survival of mice after treatment in animal model 2. The effect of *in vitro* infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and 1 day of intravenous FA and CPT-11 (genetic combination therapy, group 1) on prolongation of survival is greater than that of the conventional CPT-11, 5-FU, and FA combination chemotherapy, group 5 ($P = 0.001$).

ated by the AdLpCDIRESE1A vector/5-FC treatment within tumor cells that are not possible to safely achieve by systemic administration of 5-FU chemotherapy. In addition, we show that the toxicity of the genetic combination therapy is statistically significantly less than that seen with the conventional CPT-11, 5-FU, and FA combination chemotherapy.

Mice that were given the genetic combination therapy had a tumor volume that was almost one-fourth that seen in the mice treated with the conventional CPT-11, 5-FU, and FA combination chemotherapy ($P = 0.001$) at the end of the first month. The mice given the genetic combination therapy also had statistically significantly prolonged survival compared with the mice given the conventional combination chemotherapy ($P = 0.001$). The addition of FA to the AdLpCDIRESE1A vector and 5-FC (genetic combination therapy without CPT-11) also enhanced tumor control and survival more than the conventional CPT-11, 5-FU, and FA combination chemotherapy.

Animal model 2 was designed to test the efficacy of the vector under conditions that permitted infection of 100% of the tumor cells. This was accomplished by infecting the tumor cells with either CD-carrying vectors or wild-type adenovirus before subcutaneous inoculation of the tumor cells into the test mice. All of the mice given the genetic combination therapy were free of tumor nodules, whereas six of seven mice given AdLpCDIRESE1A + CPT-11 (intravenously) + FA (intravenously), which is the genetic combination therapy without 5-FC, developed tumor nodules.

One of the major limiting factors of the conventional interferon regulatory factor combination chemotherapy is its gastrointestinal toxicity. Importantly, there was a statistically significantly decreased incidence of diarrhea in the animals treated with the genetic combination therapy as compared with conventional CPT-11, 5-FU, and FA combination chemotherapy. We generated this reduction in toxicity and increase in efficacy of the regimen by using conditionally replication-competent adenoviral vectors that are tumor specific in the delivery of 5-FU to cancer cells, thus sparing the normal tissues of the body from the toxicity of the regimen.

The results of these studies suggest that the combination of AdLpCDIRESE1A and 5-FC system with CPT-11 and FA is more effective and less toxic than the traditional combination of CPT-11, 5-FU, and FA. Because of the limited number of tumor cells infected when this vector is injected intratumorally, the goal of clinical translation of the genetic combination therapy will be feasible when the vector has been engineered so that it only infects tumor cells and tumor vascular endothelial cells. In that case, the vector will be suitable for administration in the bloodstream, and under these conditions, it is possible that a far greater number of tumor cells can be accessed by the AdLpCDIRESE1A vector. To accomplish this, our laboratory has created adenoviral vectors carrying the CDIRESE1A transcription unit that can be targeted to the tumor cells and their vasculature, providing that the nonselective uptake of the vector by the reticuloendothelial cells has been blocked. The feasibility of taking these vectors into the clinic for use in systemic administration for tumor vascular targeting therapy is currently being studied in our laboratory.

ACKNOWLEDGMENTS

The authors would like to acknowledge Dr. Fikri Icli of Ankara University for contributions to the discussions that led to the idea of vector targeting chemotherapy.

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Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens

Yucheng Tang, Lixin Zhang, Jing Yuan, Hakan Akbulut, Jonathan Maynard, Phyllis-Jean Linton, and Albert Deisseroth

Our goal in the present work was to characterize the multiple steps involved in overcoming the anergy that exists in tumor hosts to tumor-associated antigen (TAA). Our studies showed that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector resulted in secretion of the TAA/ecdCD40L protein for at least 10 days from infected cells. Binding of the TAA/ecdCD40L protein to dendritic cells (DCs) resulted in the induction of CCR-7 chemokine receptor expression and cytokine release. This was followed by migration of the DCs to regional lymph nodes. Tetramer staining, enzyme-linked immunospot (ELISPOT) assay, and cytotoxicity assay all showed that the Ad-sig-TAA/ecdCD40L vector increased the levels of splenic CD8$^+$ T cells specific for the 2 TAAs (human MUC1 [hMUC1] and HPV E7) tested. Vaccination with the Ad-sig-hMUC1/ecdCD40L vector suppressed the growth of hMUC1 antigen–positive tumor cells in 100% of the test mice that were previously anergic to the hMUC1 antigen. These data suggest that Ad-sig-TAA-ecd/ecdCD40L vector injections may be of value in treating the many epithelial malignancies in which TAA-like hMUC1 is overexpressed. (Blood. 2004;104:2704-2713)

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Introduction

We previously reported that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector can overcome the anergy in tumor hosts against tumor-associated antigen (TAA).[1] Dendritic cells (DCs) are specialized cells of the immune system responsible for the initiation and regulation of cellular and humoral responses. The ability of DCs to regulate immunity is dependent on DC maturation. In the absence of costimulatory molecule expression on the DC surface, the presentation of TAA to naive T cells can lead to T-cell anergy caused by the induction of apoptosis in the T cells.[2]

Human DCs require multiple activation signals for the efficient generation of tumor antigen–specific T lymphocytes.[3,4] These changes endow DCs with the ability to costimulate antigen-specific CD8$^+$ and CD4$^+$ T-cell responses and to foster CD8$^+$ T-cell differentiation into cytotoxic lymphocytes (CTLs).[5,6] The fact that antigen-loaded DCs can generate antitumor immune responses capable of eradicating established tumors in vivo has been documented in a number of animal tumor models. Strategies for loading DCs with TAA include the pulsing of tumor cell RNA into DCs, the mixing of tumor cell lysates with DCs, and the in vitro addition of recombinant peptides of proven binding capability to DCs.[7-13] DC vaccination leads to tumor regression in selected patients with advanced cancer, but the weight of clinical trial data suggests that in vivo activation and tumor antigen loading of DCs might provide an advantage over in vitro activation strategies.

To develop an in vitro strategy of activation and tumor antigen–loading of DCs with which to overcome anergy to TAA, we built on the oral DNA vaccine/interleukin-2 (IL-2) targeting strategy of Xiang et al[14] to create an adenoviral vector (Ad-sig-TAA/ecdCD40L) vaccine. The Ad-sig-TAA/ecdCD40L adenoviral vector encodes a secretable (sig) form of a TAA fused to the extracellular domain (ecd) of the CD40 ligand (CD40L). The ecd of CD40L contains all the sequences necessary to form a functional trimeric CD40L.[15] Our previous studies with this vector show that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector induced immune resistance to the growth of TAA-positive cancer cells for more than 1 year.[1]

In the present work, we sought to characterize the multiple steps through which the Ad-sig-TAA/ecdCD40L vector induces an immune response to TAA in anergic animals. As shown in Figure 1A, this involves secretion of the TAA/ecdCD40L protein from the Ad-sig-TAA/ecdCD40L vector–infected cells near the subcutaneous injection site for more than 10 days. Binding of the TAA/ecdCD40L protein to the DCs resulted in activated cytokine release, increased levels of the CCR-7 chemokine, and increased membrane levels of the CD80 and CD86 receptors. This induced migration of DCs, which displayed TAA peptides on their surface major histocompatibility complex (MHC) class I molecules, and resulted in increases in the number of TAA-specific CD8$^+$ T cells competent to recognize and kill cancer cells bearing the TAA.[7,16]

We studied 2 types of TAA in this vector vaccination strategy: the human papillomavirus (HPV) E7 foreign antigen, which has been shown to be a strong stimulus of the cellular immune response,[17-20] and the ecd of the human Mucin-1 (hMUC1) self-antigen, which is expressed focally at low levels on normal epithelial cellular surfaces.[21-24] The MUC1 antigen is expressed at high levels diffusely in neoplastic epithelial mucosal cells, thereby

From the Sidney Kimmel Cancer Center, San Diego, CA.

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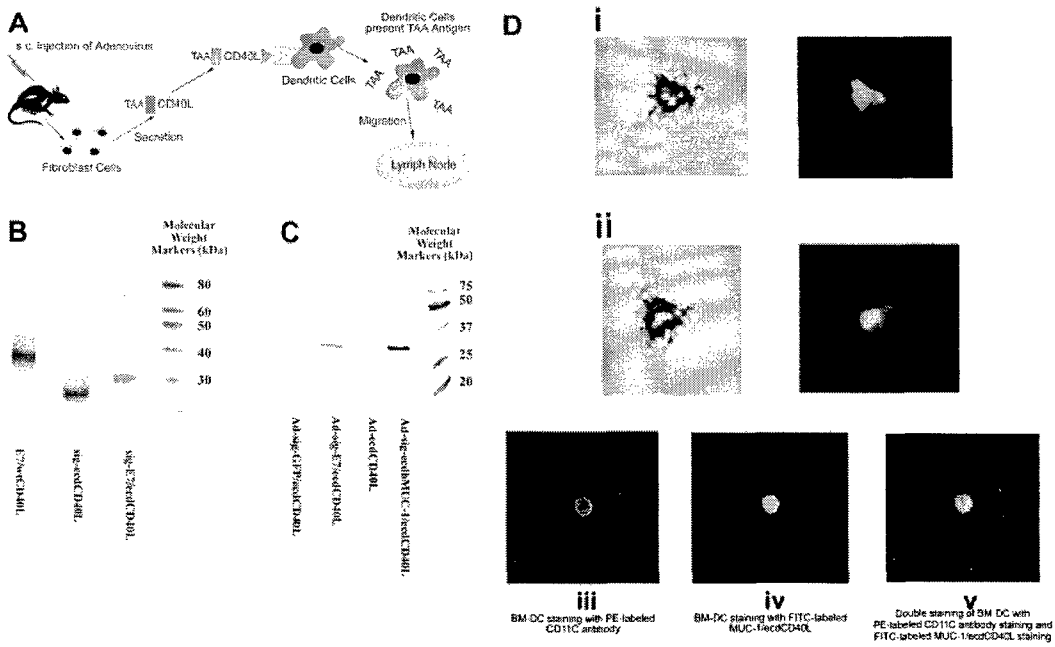

Figure 1. TAA/ecdCD40L protein produced by Ad-sig-TAA/ecdCD40–infected cells binds to DCs. (A) Proposed mechanism for induction of immune response by the Ad-sig-TAA/CD40L vector. Injecting Ad-sig-TAA/ecdCD40L induces in vivo activation and tumor-antigen loading of DCs, migration of the DCs to regional lymph nodes, and activation of $CD8^+$ cytotoxic T cells, which are specific for cells carrying the tumor antigen. (B) In vitro expression of the E7/ecdCD40L transcription unit. Plasmid expression vectors encoding the nonsecretable E7/wtCD40 ligands (lane 1), the secretable ecd of the CD40 ligand (sig-ecdCD40L) alone (lane 2), and the secretable sig-E7/ecdCD40 ligand protein (lane 3) produced in a cell-free transcription/translation system are as predicted: lane 1, E7/wtCD40L is 39 kDa; lane 2, sig-ecdCD40L is 22 kDa; and lane 3, sig-E7/ecdCD40L is 32 kDa. Molecular weight markers are in the extreme right lane. (C) Western blot analysis of the expression of E7/ecdCD40L protein in 293 cells. Molecular weights of the TAA/ecdCD40L proteins produced from 293 cells infected by the Ad-sig-TAA/ecdCD40L vectors adenoviral vectors were as predicted: lane 1, lysates from cells infected with the Ad-sig-GFP/ecdCD40L vector; lane 2, lysates from cells infected with the Ad-sig-E7/ecdCD40L vector; lane 3, lysates from cells infected with the Ad-sig-ecdCD40L vector; and lane 4, lysates from the Ad-sig-ecdhMUC1/ecdCD40L vector. Molecular weight markers are in the extreme right lane. (D) Secretory form of TAA/ecdCD40L binds in vitro to DCs. Bone marrow–derived DCs were fractionated to 78% purity. (i-ii) FITC-labeled E7/ecdCD40L recombinant proteins released from Ad-sig-E7/ecdCD40L–infected 293 cells were incubated with bone marrow–derived DCs. Cells were portioned with light microscopy (left panels) to demonstrate the morphology of the DCs and then with fluorescence microscopy (right portion panels) to detect the binding of the fluoresceinated proteins. (i) DCs incubated with FITC-labeled proteins from the supernatant of cells infected with the Ad-sig-E7/ecdCD40L. (ii) DCs incubated with FITC-labeled proteins from the supernatants of cells infected with the Ad-sig-ecdCD40L vector. (iii-v) Proteins released from Ad-sig-ecdhMUC-1/ecdCD40L-infected 293 cells were fractionated on a Nickel column to purify the His-tagged ecdhMUC-1/ecdCD40L proteins. These proteins were fluorescein labeled, as outlined in "Materials and methods." FITC-labeled ecdhMUC-1/ecdCD40L proteins and a PE-conjugated rat antimouse CD11C antibody were added to the purified DCs. (iii) Cells exposed to a laser excitatory for phycoerythrin. (iv) Cells exposed to a laser excitatory for FITC. (v) Overlay of the images from subpanels iii and iv. A Nikon Eclipse TE-2000-U microscope, which was equipped with a Perkin Elmer UltraView R55 spinning disk confocal attachment, was used at 20 × N.A. 0.5. Adobe Photoshop was the software used.

disrupting the regulation of anchorage-dependent growth, which leads to metastases.[22,23] The MUC1 antigen is a self-protein overexpressed in carcinomas of the breast, ovary, lung, prostate, colon, and pancreas, among other carcinomas.[21] Overexpression in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases.[22] Although non-MHC–restricted cytotoxic T-cell responses to MUC1 have been reported in patients with breast cancer,[23] hMUC1 transgenic mice (MUC1.Tg) have been reported to be unresponsive to stimulation with hMUC1 antigen.[24]

Our results show that immunizing hMUC1 transgenic mice, which are anergic to the hMUC1 antigen,[24] with the Ad-sig-hMUC1/ecdCD40L vector induces a $CD8^+$ T cell–dependent systemic T-helper 1 (T_H1) immune response that is antigen specific and HLA restricted and that overcomes the block in proliferation that exists in T cells in anergic hosts. Vaccination increases the frequency of hMUC1-specific T cells in the spleens of injected mice. This response requires the Ad-sig-ecdhMUC1/ecdCD40L adenoviral vector and cannot be produced by subcutaneous injection of the hMUC1/ecdCD40L protein alone. Using a similar vector system, but with the E7 antigen in place of the hMUC1 antigen, we showed that the Ad-sig-E7/ecdCD40L vector injection induced immune responses against E7-positive TC-1 tumor cells in 100% of the injected mice for up to 1 year. These results suggest that Ad-sig-TAA/ecdCD40L vector injections induce a memory cell response against TAA-positive tumor cells without the need for additional cytokine boosting treatments.

Materials and methods

Mice and cell lines

Six- to 8-week-old C57BL/6 mice were purchased from Harlan. MUC1 transgenic mice-C57/BL6/human MUC1[24] were obtained from Dr S. Gendler of Mayo Clinic Scottsdale and were bred on site.

Construction of recombinant adenoviruses

The *E7/ecdCD40L* fusion gene was constructed by ligating the amino terminal end of the ecd of CD40L to an octapeptide linker (NDAQAPKS), which was linked in turn to the carboxyl terminal end of a TAA, the amino terminal end of which was linked to a secretory signal sequence. The oligonucleotide for E7 was 5′-TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT GGA G AT ACA CCT AC-3′ and 5′-CCG CTC GAG TGG TTT CTG AGA ACA GAT GGG GCA C -3′. This oligonucleotide was cloned to the pcDNA3TOPO vector. Coding sequences for the full-length mouse CD40 ligand were generated by using the following primers: 5′-GAGAC CTC GAG AAC GAC GCA CAA GCA CCA AAA AGC ATG ATA GAA ACA TAC AGC CAA C-3′ and 5′-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3′. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection, Manassas, VA). Polymerase chain reaction (PCR) conditions are as per protocol from Tgo

DNA polymerase kit (Roche Diagnostics, Mannheim, Germany): 94°C for 3 minutes, 25 cycles at 94°C for 30 seconds, 56°C for 45 seconds,72°C for 45 seconds, and 1 cycle at 72°C for 7 minutes. The PCR fragment was inserted into the plasmid pcDNA3-E7 after restriction endonuclease digestion with *Xba*I (TCTAGA) and *Xho*I (CTCGAG). This vector was named pCDNA3CE7/wtCD40L. The E7/wt encoding DNA was cut from pCDNA3CE7/wtCD40L using *Hin*dIII-*Xba*I restriction endonuclease digestion that was then inserted into pShuttle-cytomegalovirus (CMV) downstream of the CMV promoter. This plasmid is designated pShuttle-E7/wtCD40L.

The ecdCD40L fragment for pShuttle-ecdCD40L was generated by PCR encoding the mouse immunoglobulin G (IgG) κ chain by 4 rounds of PCR amplification (first round, primers 1 and 5; second round, primers 2 and 5; third round, primers 3 and 5; fourth round, primers 4 and 5). Primers were as follows: (1) 5′-CTG CTCTGG GTT CCA GGT TCC ACT GGT GAC AAG GTC GAA GAG GAA GTA AAC C-3′; (2) 5′-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT G-3′; (3) 5′-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC3′; (4) 5′-ACG ATG GAG ACA GAC ACA C TC CTG CTA TGG GTA CTG CTG-3′; (5) 5′-CCG CGC CCC TCT AGA ATC AGA GTT TGA GTA AGC CAA AAG-3′.

The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). PCR conditions are per protocol from Tgo DNA polymerase kit (Roche Diagnostics). Conditions are the same as given earlier in this section. Fragments of ecdCD40L were cloned into the pcDNA3.1TOPO vector (Invitrogen, Carlsbad, CA), then cut from the pCDNA3-hMUC1/ecdCD40L vector using *Hin*dIII-*Xba*I restriction endonuclease digestion and inserted into pShuttle-CMV downstream of the CMV promoter and named pShuttle-ecdCD40L.

A transcription unit that included DNA encoding the signal sequence of the mouse IgG κ chain gene upstream of DNA encoding hMUC-1 was generated by PCR using plasmid pcDNA3-hMUC-1 (gift of O.J. Finn, University of Pittsburgh School of Medicine, PA) and the following primers. DNA encoding the mouse IgG κ chain METDTLLLWVLLLWVPGSTGD (single-letter amino acid code) was prepared by PCR amplification to generate the full 21–amino acid mouse IgG κ chain signal sequence: (1) 5′-CCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG-3′; (2) 5′-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC-3′; (3) 5′-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC GAT G -3′; (4) 5′-GGT TCC ACT GGT GAC GAT GTC ACC TCG GTC CCA GTC-3′; (5) 5′-GAG CTC GAG ATT GTG GAC TGG AGG GGC GGT G-3′. K/hMUC-1 with the upstream κ signal sequence was generated by 4 rounds of PCR amplification (first round, primers 4 and 5; second round, primers 3 and 5; third round, primers 2 and 5; fourth round, primers 1 and 5). PCR conditions are the same as given earlier in this section. The hMUC-1 encoding DNA was cloned into the pcDNA3.1TOPO vector (Invitrogen) forming pcDNA-hMUC-1. A pair of PCR primers was designed for ecdCD40L without the cytoplasmic and transmembrane domains: 5′-CCG CTC GAG AAC GAC GCA CAA GCA CCA AAA TCA AAG GTC GAA GAG GAA GTA -3′; 5′-GCG GGC CCG CGG CCG CCG CTA GTC TAG AGA GTT TGA GTA AGC CAA AAG ATG AG-3′. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). PCR conditions are as per protocol from the Tgo DNA polymerase kit (Roche Diagnostics), which are the same as earlier in this section. The PCR fragment was inserted into the plasmid pcDNA-hMUC-1 after restriction endonuclease digestion with *Xba*I (TCTAGA) and *Xho*I (CTCGAG). This vector was named pCDNA3-hMUC1/ecdCD40L. The hMUC1/ecdCD40L encoding DNA was cut from the pCDNA3-hMUC1/ecdCD40L vector using *Hin*dIII-*Xba*I restriction endonuclease digestion and was inserted into pShuttle-CMV downstream of the CMV promoter. The plasmid is designated pShuttle-hMUC1/ecdCD40L.

Coding sequences for the full-length mouse CD40L were generated by using the following primers: 5′-GAG ACC TCG AGA ACG ACG CAC AAG CAC CAA AAA GCA TGA TAG AAA CAT ACA GCC AAC-3′ and 5′-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3′. The CD40L template is the plasmid pDC4mCD40L (American Type Culture Collection). PCR conditions as per protocol from the Tgo DNA polymerase kit (Roche Diagnostics) are the same as given earlier in this section. Using PCR methods, in some vectors, we added the mouse HSF1 trimer domain between MUC-1 and CD40L and a His tag at the end of the CD40L. Fragments of the TAA/CD40L fusion were inserted downstream of the CMV promoter in the pShuttleCMV expression vector using the *Xho*I and *Xba*I restriction sites. The ecd of the CD40L and the full-length-wtCD40L was amplified by PCR primers and cloned into the pShuttleCMV plasmid using the *Hin*dIII and *Xba*I restriction endonuclease sites. Recombinant adenoviral vectors were generated using the AdEasy vector system.[25]

All populations of vector particles used in the experiments described in this paper were shown to contain fewer than 5 replication-competent adenoviral particles (RCAs) per 1×10^{10} viral particles (VPs).

Western blotting and in vitro expression of the E7/ecdCD40L transcription unit

Western blotting and in vitro cell-free transcription/translation were used to analyze protein expression from the vector transcription units as described previously.[30] The coupled in vitro transcription–translation system of reticulocyte lysate (RRL) (TNT kits; Promega, Madison, WI) was used to synthesize the protein products of the transgenes of the following vectors: Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L (where wt indicates the full-length or wild-type *CD40L* gene), Ad-sig-ecdCD40L, Ad-wtCD40L, and Ad-sig-ecdhMUC1/ecdCD40L. The protein cell lysate derived from 293 cells infected by each adenoviral vector described in the preceding sentence at a multiplicity of infection (MOI) of 40 was fractionated on a 10% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk for 2 hours at room temperature, the membrane was probed with an antibody against the specific mouse CD40L (mCD40LM; eBioscience, San Diego, CA) in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.5% Tween 20) in the presence of 2% bovine serum albumin (BSA) overnight. After 4 washes with TBS-T buffer, the blot was incubated with a goat antihamster alkaline phosphatase–conjugated antibody (Jackson ImmunoResearch, Bar Harbor, ME) for 1 hour. Immunoreactive bands were visualized on membranes by using the ProtoBlot II AP system (Promega).

Assay for binding of the TAA/CD40L protein to DCs

DCs were derived from incubation of bone marrow mononuclear cells in granulocyte macrophage–colony-stimulating factor (GM-CSF) and IL-4 for 7 days, followed by purification to a purity of 78% DCs. The TAA/CD40L proteins were generated by exposing 293 cells to either the Ad-sig-E7/ecdCD40L vector (Figure 1Di-ii) or the Ad-sig-ecdhMUC-1/ecdCD40L(His-tagged) vector (Figure 1Diii-v). In Figure 1Di-ii, no purification of the proteins was carried out, whereas in panel C, nickel column purification of the ecdhMUC-1/ecdCD40L proteins was carried out. The TAA/CD40L proteins were fluorescently labeled with the Fluoreporter fluorescein isothiocyanate (FITC)–protein labeling kit (Molecular Probes), added to the DCs at a final concentration of 10 μg/mL, and incubated for 30 minutes. Cells were then washed 3 times with cold medium, fixed with 1% paraformaldehyde, and observed under a fluorescence microscope.

Assay for activation of bone marrow–derived DCs

DCs were incubated with the supernatant from 293 cells infected by Ad-sig-TAA/ecdCD40L adenoviral vectors, and then plated in 24-well plates at 2×10^5 cells/mL. After incubation for 24 hours and 48 hours at 37°C, the supernatant fluid (1 mL) was harvested and centrifuged to remove debris. The level of murine IL-12 or interferon-γ (IFN-γ) released into the culture medium from vector-infected cells was assessed by enzyme-linked immunoadsorbent assay (ELISA), using mouse IL-12 p70 or IFN-γ (R&D Systems, Minneapolis, MN), respectively. Bone marrow cells were incubated for 5 days in GM-CSF and IL-4. DCs were purified with the SpinSep Mouse Dendritic Cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada). Forward and side scatter analyses of the populations before and after fractionation are given in Figure 1B-C. We then stained the bone marrow–derived DCs before and after fractionation with phycoerythrin (PE)–labeled CD11c antibody, incubating nonenriched and enriched cells for 10 minutes on ice with 5% normal rat serum to block the nonspecific

background before adding fluorochrome-conjugated antibodies. Then we stained DC fractions with PE-labeled CD11c antibody.

Detection of CCR-7 mRNA by RT-PCR

Total RNA extracted from DCs was analyzed for CCR-7 mRNA as described previously.[26] Primers for detecting CCR7 and the GAPDH control were as follows: for CCR7 sense, 5′-TCC TCC TAA TTC TTC CCT TC-3′; for CCR7 antisense, 5′-AAA CTC ATA GCC AGC ATA GG-3′); for GAPDH sense, 5′-TTG TGA TGG GTG AAC CAC-3′; and for GAPDH antisense, 5′-CCA TGT AGG CCA TGA AGT CC-3′. Expected sizes of the amplified fragments were 400 bp for CCR7 and 525 bp for GAPDH. Amplified samples were resolved on ethidium bromide–stained agarose gels. Total cellular RNA was extracted using the Trizol reagent (Life Technologies, Burlington, ON, Canada). Reverse transcription–polymerase chain reaction (RT-PCR) was performed on 5 μg RNA for the reverse transcription reaction. Half of each cDNA product was used to amplify CCR-7 and GAPDH.

DC migration assays

Bone marrow–derived DCs were loaded with the carboxyfluorescein diacetate succinimidyl ester (CFDA SE) supravital dye for 15 minutes at 37°C (Molecular Probes, Eugene, OR). Rinsed DCs were mixed with each recombinant adenoviral vector at an MOI of 200 and were injected into the left flank of the test mouse. Three days later, axillary lymph nodes draining the region of the injection site for the DCs were removed, and frozen tissue sections were made and observed under the fluorescence microscope.

Immunohistochemical staining

Immunized mice were killed 3 and 10 days after injection of the Ad-sig-E7/ecdCD40 vector. Skin at each site of subcutaneous vector injection was subjected to biopsy, embedded in optimum cutting temperature (OCT) solution, and cut into 5-μm sections. Slides were incubated with rat anti-CD40L antibody (eBioscience) and exposed to biotinylated goat anti–rat IgG antibody (1:200 dilution) and avidin-biotin complex (Vector Laboratories, Burlingame, CA). Stained slides were then mounted and studied under a fluorescence microscope.

Tetramer and ELISPOT assays

PE-labeled H-2D^b tetramers containing HPV16 E7$_{49\text{-}57}$ peptide (RAHYNIVTF) were purchased from Beckman Coulter (Hialeah, FL) and were used for the fluorescence-activated cell sorter (FACS) analysis of peptide-specific CTL immunity. Tetramer-positive and CD8$^+$ cells are shown as percentages of total spleen cells. The presence of E7- and hMUC1-specific effector T cells in the immunized mice was also assessed by carrying out enzyme-linked immunospot (ELISPOT) assays, as previously described.[27]

Cytotoxicity assay

E7-positive TC-1 target cells or LL2/LL1hMUC1-positive target cells (5×10^3) were incubated with splenic mononuclear cells (effector cells) at varying effector-target ratios (100:1, 20:1, and 5:1) for 4 hours at 37°C, in culture media containing 5% fetal bovine serum (FBS). Effector cells had been prestimulated with the TAA-positive cancer cells for 5 days in vitro before the in vitro cytotoxicity assay. Cell-mediated cytotoxicity was determined using a nonradioactive lactate dehydrogenase (LDH) release assay. Student unpaired *t* test was used to determine differences among the various groups in cytotoxicity assays. Statistical significance was defined by the *P* less than .05 level.

In vivo efficacy experiment in mouse model

Mice (5 or 10 per group) were vaccinated through subcutaneous injection with 1×10^8 plaque-forming units (pfus) of the Ad-sig-TAA/ecdCD40L, Ad-TAA, Ad-TAA/wtCD40L, Ad-sig-CD40L, Ad-wtCD40L, or Ad-sig-ecdhMUC1/ecdCD40L vectors. One week later, mice were boosted with the same adenoviral vector regimen as the first vaccination. One week after the last vaccination, mice were challenged by subcutaneous injection of 5×10^5 TAA-positive cancer cells. Tumor volumes were measured in centimeters by caliper, and the volumes were calculated as tumor volume = length × (width^2)/2 (this assumes an elliptical shape).

Analysis of p44/p42 mitogen-activated protein kinase and SAPK/JNK phosphorylation

Western blot analysis of p44/p42 and SAPK/JNK was carried out with kits (no. 9100 for p44/p42 and no. 9250 for SAPK/JNK) from New England Biolabs (Beverly, MA). Responder splenocytes were isolated from vaccinated mice and enriched in CD8$^+$ cells using a murine CD8 T-cell enrichment kit (catalog 13033; StemCell Technologies, Vancouver, BC, Canada). Bone marrow–derived DCs were infected with Ad-sig-ecdlMUC1/ecdCD40L for 2 hours, then washed with phosphate buffered saline and incubated for 48 hours.[28] Responder cells were mixed in a 1:1 ratio with Ad-sig-ecdhMUC1/ecdCD40L infected antigen-presenting cells (APCs), and Western blot analysis was performed at the indicated time points.

Statistics

All parameters were analyzed using Student *t* test or analysis of variance (ANOVA), followed by the Scheffé procedure for multiple comparisons as post hoc analysis. All data shown are presented as mean ± SEM.

Results

TAA/ecdCD40L protein binds to DCs

Cell free–coupled transcription/translation and Western blot analysis of the E7/ecdCD40L, E7, ecdCD40L, E7/wtCD40L, and wtCD40L proteins were used to study the molecular weights of the proteins produced in cells infected by the Ad-sig-E7/ecdCD40L, Ad-sig-E7, Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-wtCD40L vectors, respectively. As shown in Figure 1B-C, the molecular weights of these proteins are those predicted.

We then collected the TAA/ecdCD40L proteins from vector-infected 293 cells and labeled these proteins with fluorescein (see "Materials and methods"). These proteins were then incubated in vitro with bone marrow–derived DCs (fractionated to 78% purity) for 30 minutes at 4°C. The DCs were washed and portioned once using light microscopy and again using fluorescence microscopy. As shown in Figure 1Di-ii, the secretable form of E7/ecdCD40L can bind to the DCs.

A second experiment was carried out in which 293 cells were infected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (His tag present), and the proteins were fluorescein labeled after purification of the MUC-1/ecdCD40L proteins on a Nickel column. The cells were exposed to a PE-conjugated anti-CD11C antibody and to the FITC-conjugated ecdhMUC-1/ecdCD40L proteins. The results (Figure 1Diii-v) show that the DCs bind the ecdhMUC-1/ecdCD40L proteins.

E7/ecdCD40L protein can be detected in vivo for up to 10 days in vivo after subcutaneous injection of the Ad-sig-E7/ecdCD40L vector

We then sectioned the skin at the site of intradermal injection of the Ad-sig-E7/ecdCD40L vector to determine when the secretable sig-E7/ecdCD40L protein was released from vector-infected cells. We double stained these sections with an FITC-labeled antibody to the CD40L (CD154), which stained green (Figure 2A), and DAPI, which stained the nuclear DNA blue (Figure 2A). As indicated in Figure 2A, double staining showed that the TAA/CD40L protein bound in vivo to cells near the vector-infected cells for up to 10 days after subcutaneous injection with the Ad-sig-E7/ecdCD40L vector, which carried the secretable TAA/ecdCD40L transcription unit. In contrast, a lower level of double-stained positive cells was

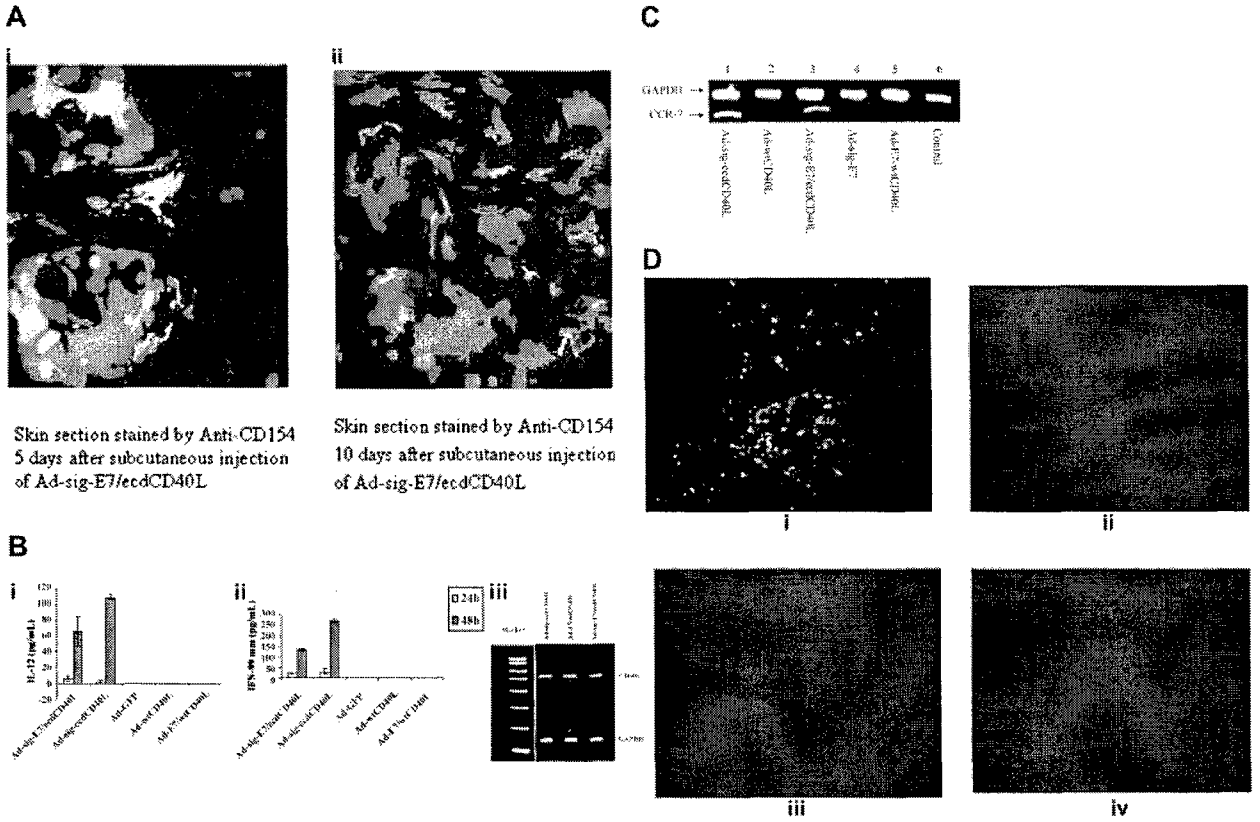

Figure 2. TAA/ecdCD40L protein from Ad-sig-TAA/ecdCD40L vector–infected cells binds to and activates DCs, which induce migration to regional lymphoid tissue. (A) Injection of the Ad-sig-E7/ecdCD40L vector generates the release of the E7/ecdCD40L protein around the vector injection site for up to 10 days. Skin section stained by anti-CD154 and DAPI 5 days (i) and 10 days (ii) after injection of the Ad-sig-E7/ecdCD40L vector. (B) Bone marrow–derived DCs release IL-12 and IFN-γ after exposure to the Ad-sig-E7/CD40L Vector. IL-12 (i) or IFN-γ (ii) released by vector-infected DCs into the supernatant medium was measured by ELISA in DCs stimulated for 24 hours (light gray bars) and 48 hours (dark gray bars) with the adenoviral vectors Ad-sig-E7/ecdCD40L, Ad-ecdCD40L, Ad-GFP, Ad-wtCD40L, and AD-E7/wtCD40L. (iii) Semiquantitative RT-PCR reaction was used to measure the levels of E7/CD40L RNA in 293 cells exposed to the Ad-sig-eE7/ecdCD40L vector or the Ad-E7/wtCD40L vector. 293 cells were infected with the vectors Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-sig-E7/ecdCD40L at an MOI of 10. Then the RNA was isolated and PCR was carried out with primers specific for E7/CD40L mRNA. The cDNA generated was then fractionated on a molecular-weight gel. The electrophoretic species corresponding to the predicted molecular weight of the PCR product from the E7/CD40L template is indicated in the right-hand margin of the gel by the CD40L label. Electrophoretic mobility of a PCR cDNA product using the same RNA but primers specific for GAPDH (loading control) is indicated in the right-hand margin by glyceraldehyde phosphate dehydrogenase (GAPDH). (C) Up-regulation of CCR-7 mRNA in DCs exposed to the Ad-sig- E7/ecdCD40L vector. Lane 1: the Ad-sig-ecdCD40L vector. Lane 2: the Ad-wtCD40L vector. Lane 3: the Ad-sig-E7/ecdCD40L vector. Lane 4: the Ad-E7 vector. Lane 5: the Ad-E7-wtCD40L vector. Lane 6: uninfected cells (control). (D) In vivo study of migration of DCs to regional lymph nodes after loading of DCs with CFDA SE dye and infection with the Ad-sig-E7/ecdCD40L vector. Bone marrow–derived DCs were loaded in vitro with the CFDA SE supravital dye, exposed in vitro to the following vectors at an MOI of 200. (i) Ad-sig-E7/ecdCD40L. (ii) Ad-ecdCD40L. (iii) Ad-E7/wtCD40L. (iv) Ad-wtCD40L. DCs were then injected subcutaneously into the hind flanks of the test mice. Two days later, regional lymph nodes were dissected and frozen sections were studied under a fluorescence microscope. Color micrographs were obtained.

observed in the epidermis 3 days after injection of the Ad-E7/wtCD40L, which contained a nonsecretable CD40L transcription unit (data not shown).

Activation of DCs by the Ad-sig-E7/ecdCD40L vector

As shown in Figure 2Bi, there was a statistically significant increase in the level of induction of IL-12 production after in vitro exposure of the DCs to the supernatant of Ad-sig-E7/ecdCD40L vector–infected 293 cells. This vector carried a transcription unit encoding a secretable TAA/CD40L protein as in Figure 1. The results were compared with vectors encoding a nonsecretable TAA/CD40L protein, such as the Ad-E7/wtCD40L vector ($P < .0001$). IL-12 (6 ± 3 pg/2×10^5 cells per milliliter per 24 hours or 66 ± 18 pg/2×10^5 cells per milliliter per 48 hours) was produced by DCs exposed to the Ad-sig-E7/ecdCD40L vector supernatant, whereas exposing DCs to the Ad-E7/wtCD40L vector supernatant resulted in no measurable IL-12 at 24 hours or 48 hours.

Similarly, there was a statistically significant increase in the IFN-γ released from DCs exposed to the supernatant from the Ad-sig-E7/ecdCD40L vector–infected cells: 24 ± 3 pg in the first 24 hours and 132 ± 6 pg during the next 24 hours, compared with 0 pg released from DCs exposed to supernatant from 293 cells infected with nonsecretable CD40L vectors or other control vectors (Figure 2Bii). These experimental data suggest that the TAA/ecdCD40L fusion protein secreted from the Ad-sig-TAA/ecdCD40L-infected cells bound to the CD40 receptor on DCs to generate the observed effect on cytokine release.

Differences between the cytokine release induced in bone marrow–derived DCs exposed to the supernatant from 293 cells infected with CD40L secretable or nonsecretable transcription units could be attributed to the E7/CD40L RNA levels generated by the Ad-sig-E7/ecdCD40L (encoding the secretable E7/CD40L protein) compared with the Ad-E7/wtCD40L (encoding the nonsecretable E7/CD40L protein). Another possibility is that one vector encodes a secretable or a nonsecretable protein. To test this question, RNA was extracted from 293 cells that had been infected by either the Ad-sig-E7/ecdCD40L vector or the Ad-E7/wtCD40L vector at an MOI of 10. The cDNA was synthesized by using the superscript first-strand system (Invitrogen, Carlsbad, CA). RT-PCR was performed using 5 μg total RNA extracted from the vector-infected cells and the reverse transcription reaction with a random primer. The cDNA product was split into 2 halves; one half was

used as a template for a PCR reaction with primers specific for the E7/CD40L cDNA, and the other half was used to prime a PCR reaction with primers specific for GAPDH as a control. Results shown Figure 2Biii, indicate no difference in the E7/CD40L mRNA levels using the secretable or the nonsecretable vectors. Thus, it appears that cytokine release is greater from bone marrow–derived DCs exposed to the supernatant from 293 cells infected with the Ad-sig-E7/ecdCD40L rather than the Ad-E7/CD40L vector because of the secretable nature of the E7/CD40L protein from the Ad-sig-E7/ecdCD40L-infected cells.

In vitro and in vivo exposure of DCs to the Ad-sig-E7/ecdCD40L vector elevates CC chemokine receptor-7 (CCR-7) expression in mature DCs and induces the migration of DCs to regional lymph nodes

On antigen exposure, DCs become activated, express CCR-7, and migrate in response to differential gradients of the chemokine ligands CCL 19 and CCL 21.[26] Therefore, we investigated the effect of exposing DCs to supernatants from Ad-sig-E7/ecdCD40L-infected 293 cells to determine whether the level of CCR-7 expression increased. As shown in Figure 2C, the level of CCR-7 mRNA in DCs increased significantly when DCs were cultured with supernatants from Ad-sig-E7/ecdCD40L or Ad-sig-E7/ecdCD40L vector–infected 293 cells.[26]

To formally test whether the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector induces migration of the DCs to the regional lymph nodes in vivo,[26] 1×10^6 DCs were loaded with the CFDA SE dye and were exposed to adenoviral vectors at an MOI of 200. Then, the dye-loaded DCs were injected into the left flanks of the C57BL/6 mice. Three days after these injections, the mice were killed, and the regional axillary lymph nodes on the side of the injection were harvested and studied for the presence of the dye-loaded DCs. As shown by the green dots visible in Figure 2Di, CFDA SE–stained DCs are detectably present in the regional lymph nodes after injection of the vector carrying the secretable E7/ecdCD40L transcription unit, whereas no other vector (Figure 2Dii-iv) was associated with detectable fluorescence-labeled DCs in the regional lymph nodes. No CFDA SE–labeled cells were observed in the nondraining, contralateral lymph nodes. One of the sections was stained with PE-labeled CD11C antibody to confirm that the green-stained cells were DCs (data not shown).

Injection of Ad-sig-E7/ecdCD40L suppresses growth of E7-positive cancer cells in syngeneic mice

To assess the effect of subcutaneous injection of the Ad-sig-E7/ecdCD40L vector on the engraftment of the E7-positive TC-1 cell line in C57BL/6 mice, we injected 1×10^8 pfu of each vector subcutaneously into each animal. Mice were vaccinated again 1

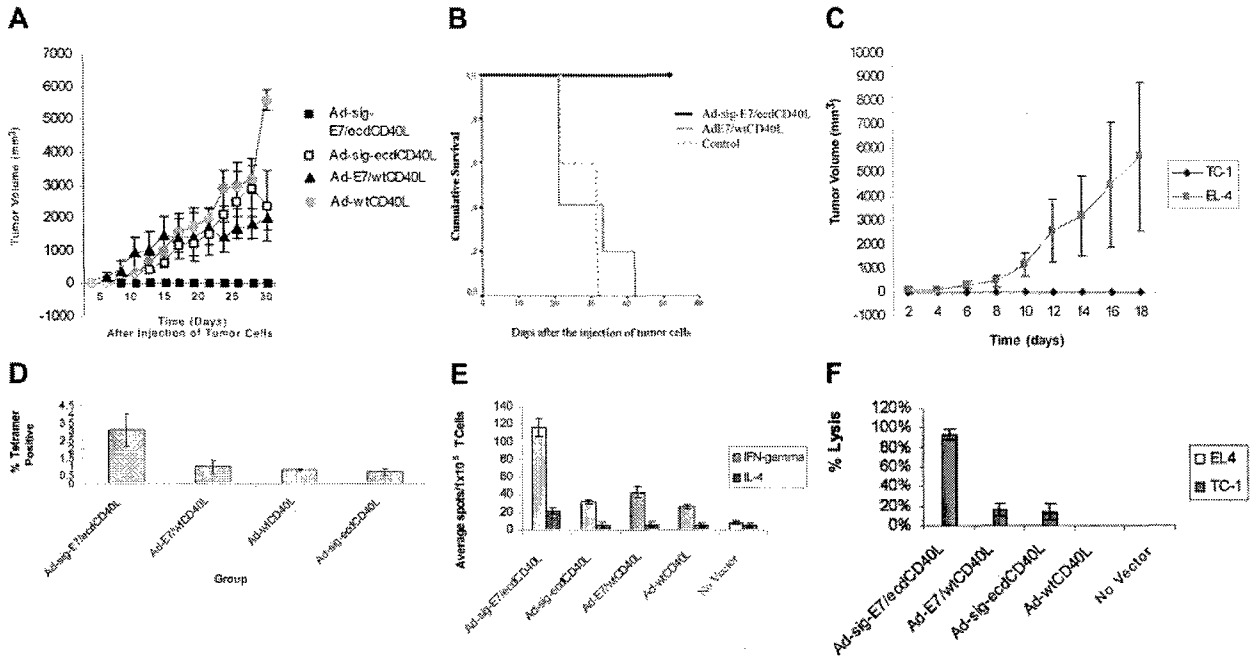

Figure 3. Mechanism of the Ad-sig-E7/ecdCD40L vector–induced suppression of the growth of E7-positive TC-1 tumor cells in C57BL/6 mice. (A) Resistance to the subcutaneous growth of 5×10^5 E7-positive TC-1 cancer cells in mice after 2 injections with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector 7 days apart. (■) Ad-sig-E7/ecdCD40L. (□) Ad-sig-ecdCD40L. (▲) Ad-E7/wtCD40L. (◎) Ad-wtCD40L. (B) Survival of mice vaccinated with Ad-sig-E7/ecdCD40L vectors. The following vectors were injected into C57BL/6 mice, after which the E7-positive TC-1 cancer cells were injected into the subcutaneous spaces of the mice: bold continuous line, mice treated with 2 subcutaneous injections 7 days apart of 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector; thin continuous line, mice treated with subcutaneous injections of the Ad-wtCD40L vector; broken thin line, control mice, which were not treated with vector injections. (C) Comparison of the effects of 2 subcutaneous injections of 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector on the in vivo growth of the E7-positive TC-1 cells (◆) and the E7-negative EL-4 cell line (▨). Sizes of the subcutaneous tumors were estimated by measuring with calipers in 2 separate orthogonal directions and then calculating the volume assuming an elliptical shape. (D) Use of tetramers to measure the level of E7-specific $CD8^+$ T cells in the spleens of Ad-sig-E7/ecd/CD40L vector–immunized C57BL/6 mice. Spleen cells were harvested 10 days after the completion of 2 subcutaneous injections 7 days apart with 1×10^8 pfu of vectors Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-wtCD40L, and Ad-sig-ecdCD40L. T cells were then analyzed for the percentage of $E7_{49\text{-}57}$ peptide-specific $CD8^+$ T-cell lymphocytes by H-$2D^b$ tetramer staining. (E) ELISPOT assay shows increase in the level of IFN-α–secreting cells in the spleen cells of mice injected subcutaneously twice (7 days apart) with 1×10^8 pfu Ad-sig-E7/ecdCD40 vector. Mice were injected twice with the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-wtCD40L. Splenic T cells taken from the mice 1 week later were analyzed by ELISPOT assay for the presence of IFN-γ. (F) Increase in the level of E7-specific CTLs in the spleens of Ad-sig-e7/ecdCD40L–injected mice. Mice were injected subcutaneously twice (7 days apart) with 1×10^8 pfu of vectors Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-sig-ecdCD40L, Ad-wtCD40L, and control (no vector injection). T cells were harvested from the spleens of the test mice 1 week after the second adenoviral vector injection and were restimulated in vitro with TC-1. After 7 days, restimulated effector cells (spleen cells exposed to TC-1 cells in vitro) were mixed at varying ratios with TC-1 (E7-positive) and EL-4 (E7-negative) target cells. Then the LDH released from the target cells was measured. No LDH was detectable from any of the mixtures of EL-4 and the restimulated effector cells isolated from the vaccinated mice, whereas significant levels of LDH were released from the TC-1 target cells when they were mixed with the restimulated effector cells isolated from the mice vaccinated with the Ad-sig-E7/ecdCD40L vector.

week later with the same vector. One week after this boost, 5×10^5 E7-positive TC-1 cells were injected subcutaneously on the backs of the C57BL/6 mice at a site different from that of the vector injections. All mice injected with the Ad-sig-E7/ecdCD40L vector remained tumor free throughout the study (up to 18 days after injection), whereas mice injected with all other vectors listed in Figure 3A, including the Ad-E7/wtCD40L vector, which did not carry a secretable TAA/CD40L transcription unit, had measurable tumors within 13 days of tumor challenge (Figure 3A).

As shown in Figure 3B, the survival of the mice injected with the Ad-sig-E7/ecdCD40L vector (bold, unbroken line at the top of the graph) and then injected with the E7-positive TC-1 cells was superior to the survival of mice injected with the Ad-E7/wtCD40L vector (thin, unbroken line), which does not encode a secretable E7/CD40L protein, or injected with no vector (thin, broken line) and then injected with the TC-1 cells.

We then tested whether inducing resistance to engraftment of the E7-positive TC-1 cells was specific for the E7 antigen. As shown in Figure 3C, subcutaneous injection of the Ad-sig-E7/ecdCD40L vector did not protect mice against the engraftment of E7-negative EL-4 cells but did protect against engraftment of the E7-positive TC-1 cells.

Mechanism of suppression of E7-positive tumor cells by Ad-sig-E7/ecdCD40L vector injections

Spleens were harvested 10 days after vector vaccination, and the percentage of $E7_{49\text{-}57}$ peptide-specific $CD8^+$ T cells was determined by H-$2D^b$ tetramer staining. As shown in Figure 3D, the level of E7 peptide–specific T cells in the spleen cells from Ad-sig-E7/ecdCD40L injected animals was increased 3 times compared with the level observed after injection with other vectors, including the Ad-E7/wtCD40L vector.

The frequency of IFN-γ– and IL-4–secreting T cells from the spleens of mice vaccinated with the various vectors was determined by ELISPOT assays.[27] As shown in Figure 3E, mice injected with the Ad-sig-E7/ecdCD40L vector had a greater number of IFN-γ–secreting T cells (117 ± 10.6 spots/1×10^5 spleen cells) than mice injected with the vector carrying the nonsecretable E7/wtCD40L transcriptional unit (26.3 ± 2.4 spots/1×10^5 spleen cells) or any of the other control vectors tested ($P \leq .05$). The number of splenic T cells producing a T_H2 cytokine (IL-4) was only (22.3 ± 3.68 spots/1×10^5 spleen cells). These data indicate that the Ad-sig-E7/ecdCD40L vector vaccination stimulates a T_H1 rather than a T_H2 immune response.

Spleen cells from mice injected with the Ad-sig-E7/ecdCD40L vector were prestimulated in vitro for 7 days with TC-1–positive cells and then mixed in a 100:1 ratio with E7-positive TC-1 cells in a cytotoxicity assay described in "Materials and methods." These studies showed that the splenic T cells from the Ad-sig-E7/ecdCD40L vector–sensitized animals lysed 90% of the TC-1 target cells (Figure 3F). In contrast, spleen cells from uninjected mice or from mice injected with the Ad-E7/wtCD40L vector lysed 0% or 20% of the target cells, respectively.

To test whether the induced cytolytic immune response was mediated through an HLA-restricted process, we added anti-MHC class I antibody or an isotype-matched control antibody to the mixture of effector spleen cells from Ad-sig-E7/ecdCD40L vector–injected mice and E7-positive TC-1 target cancer cells. Adding the anti-HLA antibody suppressed cytotoxicity to the TC-1 target cells to 10.32%, which is significantly lower than the cytotoxicity found with control antibody (76.91%).

Injection of the Ad-sig-ecdhMUC1/ecd/CD40L vector overcomes anergy to hMUC1-positive cells in mice transgenic for the *hMUC1* gene

We first exposed bone marrow–derived DCs to the Ad-sig-ecdhMUC1/ecdCD40L vector or to the Ad-sig-ecdhMUC1 vector. As shown in Figure 4A-B, the ecdhMUC1/ecdCD40L fusion protein can significantly increase the levels of IFN-gamma and IL-12 cytokines secreted from DCs harvested from hMUC1.Tg transgenic mice 48 hours after exposure to the vector. These studies suggest that the ecdhMUC1/ecdCD40L fusion protein can bind to the CD40 receptors on DCs and induce DC activation.

Testing for functional trimers of ecdhMUC1/ecdCD40L proteins induced by the Ad-sig-ecdhMUC1/ecdCD40L vector injections that can activate DCs

To formally test whether trimeric ecdhMUC1/ecdCD40L proteins are released after the infection of cells with Ad-sig-ecdhMUC1/ecdCD40L vector, we purified (using a His Tag purification kit) the ecdhMUC1/ecdCD40L protein from the supernatant of 293 cells exposed to the Ad-sig-ecdhMUC1/ecdCD40L. In this vector, an HSF1 trimer stabilization domain had been placed between the ecdhMUC1 and the ecdCD40L fragments, and a His tag was placed at the carboxyl terminal domain of the ecdCD40L protein. As shown in Figure 4C, the molecular weight of the ecdhMUC1/ecdCD40L protein under nondenaturing conditions was close to 3

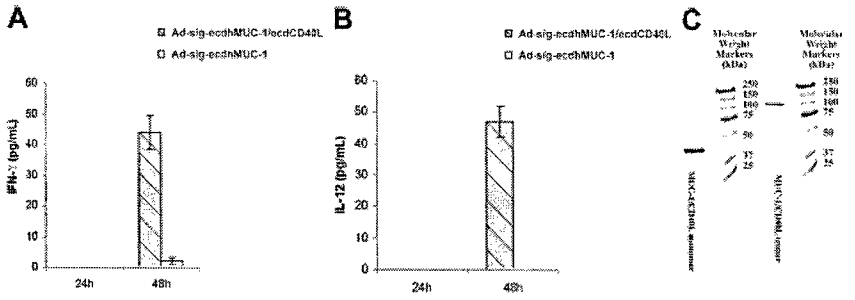

Figure 4. The ecdhMUC1 protein released from Ad-sig-ecdhMUC1/ecdCD40L vector–infected cells forms functional trimers and activates DCs. (A) Induction of IFN-γ secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector. Supernatant medium collected from DCs derived in vitro from hMUC1.Tg mice after exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector or to the Ad-sig-ecdhMUC1 vector and then analyzed for the levels of IFN-γ. (B) Induction of IL-12 secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L or the Ad-sig-ecdhMUC1 vectors. The same procedure outlined for panel A was carried out, except that the supernatant medium was analyzed for IL-12. (C) Nondenaturing gel analysis of molecular weights of the ecdhMUC1/ecdCD40L protein. A construct was created in which a His tag was placed at the carboxyl terminal end of the CD40L, and an HSF1 trimeric stabilization domain was added between the ecdhMUC1 and ecdCD40L domains. After release from vector-infected cells, the protein was purified using a His tag column, concentrated, and added to a nondenaturing gel. The protein in the lane labeled MUC1/CD40L trimer was added to the nondenaturing gel without treatment. The protein in the lane labeled MUC1/CD40L monomer was first treated with the denaturing conditions before loading on the gel. Molecular weight markers are given in the extreme right lane.

times that seen under denaturing conditions. This experiment showed that trimers could be formed by the ecdhMUC1/ecdCD40L fusion protein.

Subcutaneous injection of the Ad-sig-ecdhMUC1/ecdCD40L vector overcomes anergy for hMUC1 positive cells in mice, which are transgenic for hMUC1

As shown in Figure 5A, mice injected subcutaneously with the Ad-sig-ecdhMUC1/ecdCD40L vector (solid squares) were resistant to engraftment by the hMUC1-positive LL2/LL1hMUC1 mouse cancer cells, whereas mice vaccinated with the Ad-sig-ecdhMUC-1 vector (solid triangles) or the untreated control animals not injected with vector (solid diamonds) were not resistant to the growth of the same cells. These data show that the full chimeric hMUC-1/ecdCD40L transcription unit is needed for complete suppression of the growth of the hMUC-1 cell line in the hMUC-1.Tg mice.

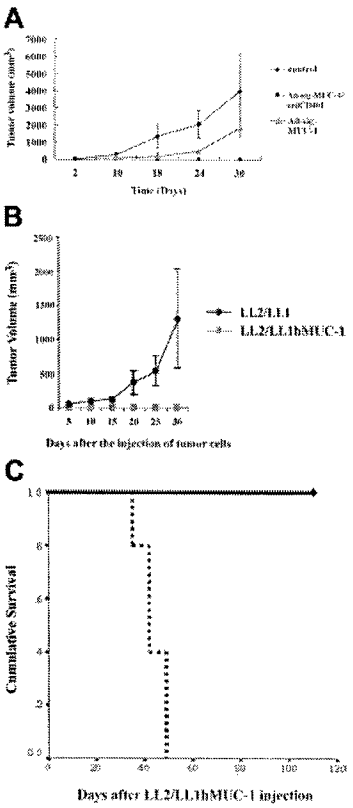

Figure 5. Effect of 2 subcutaneous injections (7 days apart) of 1 × 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L vector on the in vivo growth of the hMUC1-positive LL2/LL1hMUC1 cancer cell line in hMUC1.Tg mice. (A) Two subcutaneous injections (7 days apart) of 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector suppresses the growth of the human MUC1-positive LL2/LL1hMUC1 cancer cell line. The Ad-sig-ecdhMUC1/ecdCD40L vector or the Ad-sig-ecdhMUC-1 vector was injected twice at 7-day intervals or was not injected with any vector. One week after the second vector injection, the mice were injected with 5 × 10^5 LL2/LL1hMUC1 cancer cells, which were positive for hMUC1, and the growth of these cells was measured with calipers. (B) The Ad-sig-ecdhMUC1/ecdCD40L-induced suppression is specific for the hMUC1 antigen. hMUC1.Tg mice were injected twice subcutaneously (7 days apart) with 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector twice at 7-day intervals. One week after the second vector injection, the mice were injected with 5 × 10^5 LL2/LL1hMUC1 cells positive for the hMUC1 antigen or the same number of LL2/LL1 cells negative for the hMUC1 antigen. (C) Survival of LL2/LL1hMUC1 cell line–injected hMUC1.Tg mice that were twice (7 days apart) subcutaneously vaccinated or not vaccinated with 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector. Mice that received the injections outlined in panel A were monitored for survival after injection of the LL2/LL1hMUC1 cells. Continuous bold line indicates mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector. Broken bold line indicates mice not injected with a vector.

Mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector suppressed the growth of the hMUC1 antigen–positive LL2/LL1hMUC1 cell line, whereas this same vector did not suppress the growth of the parental cell line (LL2/LL1), which was not positive for the hMUC1 antigen (Figure 5B).This showed that the immune response was antigen specific.

As shown in Figure 5C, mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector (solid bold line at the top of Figure 5C) lived longer than did mice injected with a control vector (broken line in Figure 5C) and then injected subcutaneously with the LL2/LL1hMUC1 cell line.

Study of the cellular mechanisms through which Ad-sig-ecdhMUC1/ecdCD40L subcutaneous injections overcome anergy

Will the injection of the ecdhMUC1/ecdCD40L protein overcome anergy in the hMUC1.Tg mouse without the vector danger signal? One question is whether the subcutaneous injection of the ecdhMUC1/ecdCD40L protein would induce the cellular immune response that was seen with the Ad-sig-ecdhMUC1/ecdCD40L vector injections. As shown by the data in Figure 6A, subcutaneous injection of the ecdhMUC1/ecdCD40L protein did not induce an immune response that could protect the hMUC1.Tg mice from the growth of the LL2/LL1hMUC1 cell line. It is possible that the use of the adenoviral vector injections provide the so-called danger signal[2] necessary to induce the immune response in the hMUC1.Tg mice.

Cytokine release from vaccinated compared with nonvaccinated mice. To test whether the Ad-sig-ecdhMUC1/ecdCD40L induction of cellular immunity was mediated by CD8 T cells, the spleen T cells of the Ad-sig-ecdhMUC1/ecdCD40L vector vaccinated hMUC-1.Tg mice or the Ad-sig-ecdhMUC-1 vaccinated mice were depleted of CD4 T-cell lymphocytes with magnetic beads. As shown in Figure 6B, the CD8 T-cell lymphocytes isolated 7 days after injection from the spleens of hMUC1.Tg mice with the Ad-sig-ecdhMUC1/ecdCD40L vector released more than 2500 times the level of IFN-γ as did CD8 T cells taken from control vector–vaccinated MUC1.Tg mice and 50 times the levels of IFN-γ as did mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

Cytotoxicity assay of splenic T cells from Ad-sig-ecdhMUC1/ecdCD40L vector injected mice against LL2/LL1hMUC1 or LL2/LL1 cancer cells. Splenic T cells were collected from hMUC1.Tg mice 7 days after injection with the Ad-sig-ecdhMUC1/ecdCD40L vector or the Ad-sig-ecdhMUC-1 vector and were then exposed to the hMUC1 antigen–positive LL2/LL1hMUC1 cancer cells for 7 days. Stimulated T cells were then mixed in varying ratios with either the hMUC1-positive LL2/LL1hMUC1 cells or the hMUC1-negative LL2/LL1 cancer cells. As shown in Figure 6C, T cells from Ad-sig-ecdhMUC1/ecdCD40L vaccinated mice can specifically kill cancer cells carrying the hMUC1 antigen but not the antigen-negative cells. Moreover, the level of hMUC-1 specific cytotoxic T cells in the Ad-sig-ecdhMUC-1/ecdCD40L mice was 6 times higher than in mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

Ad-sig-ecdhMUC1/ecdCD40L vector injection overcomes resistance to expansion of hMUC1-specific T cells. Although anergic peripheral $CD8^+$ T cells can be induced to lyse target cells in an antigen-specific manner, they have been found to exhibit a block in the activation of the ERK proliferation signal transduction pathway after antigenic stimulation.[28] To determine whether CD8 cells from hMUC1.Tg mice expressed the active form of ERK1/2 on vector immunization, splenic CD8-positive T cells were obtained from noninjected hMUC1.Tg transgenic mice or mice

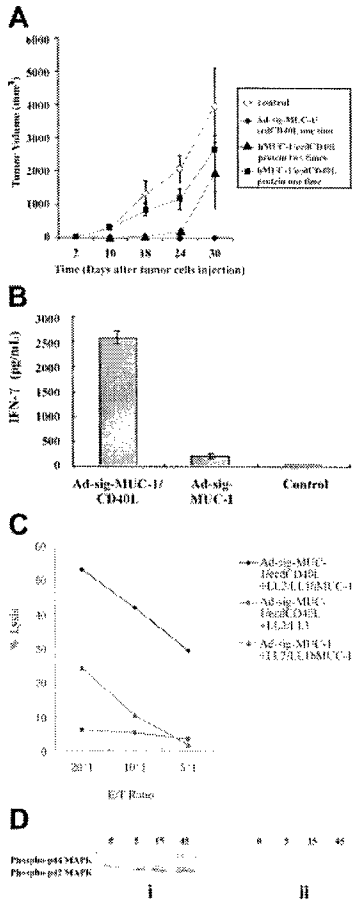

Figure 6. Mechanism of the suppressive effect of the Ad-sig-ecdhMUC1/ecdCD40L vector on induction of the immune suppression of the growth of the LL2/LL1hMUC1 cells in hMUC1.Tg mice. (A) Subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce suppression of the growth of hMUC1-positive cells, which is equivalent to that seen with 2 subcutaneous injections of 1×10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector. Five hundred thousand LL2/LL1hMUC1 cells were injected subcutaneously into the hMUC1.Tg mice. Two days after injection of the tumor cells, the ecdhMUC1/ecdCD40L protein was injected subcutaneously into hMUC1.Tg mice. (○) No protein injection. (♦) Ad-sig-ecdhMUC1/ecdCD40L vector. (▲) Two injections of the ecdhMUC1/ecdCD40L protein. (■) One injection of the ecdhMUC1/ecdCD40L protein. (B) $CD4^+$-depleted T cells from hMUC1 transgenic mice after 2 subcutaneous injections of 1×10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector secrete increased levels of IFN-γ. $CD8^+$ T cells were isolated from hMUC1.Tg mice that had been vaccinated twice with the Ad-sig-ecdhMUC1/ecdCD40L vector or with the Ad-sig-ecdhMUC-1 vector or that had been unvaccinated (labeled as control). Seven days after vaccination, $CD8^+$ cells were harvested from the spleens of the test animals and were incubated for 24 hours. The supernatant medium was analyzed for IFN-γ levels. (C) Cytotoxicity of CTLs from hMUC1.Tg transgenic mice after 2 subcutaneous injections (7 days apart) of 1×10^8 pfu of Ad-sig-ecdhMUC1/ecdCD40L vector against LL2/LL1-MUC1 hMUC1–positive cancer cells or against LL2/LL1 cancer cells negative for the hMUC1 antigen. $CD8^+$ T-cell lymphocytes were isolated from the spleens of hMUC1.Tg mice 1 week after vaccination with the Ad-sig-ecdhMUC1/ecdCD40L vector. Cells were restimulated in vitro with the LL2/LL1hMUC1 cell line for 5 days (♦) or the LL2/LL1 cell line (■). $CD8^+$ T-cell lymphocytes were also isolated from the spleens of hMUC1.Tg mice 1 week after vaccination with the Ad-sig-ecdhMUC1 vector, which was then stimulated in vitro with the LL2/LL1hMUC1 cell line (▲). Different effector/target ratios (20:1, 10:1, and 5:1) were used. The LDH released from each of these cell mixtures (ordinate) was then measured. (D) Phosphorylation of the ERK1/ERK2 proliferation pathway in CD8 T cells from hMUC1 transgenic mice after stimulation with bone marrow–derived DCs infected with the Ad-sig-ecdhMUC1/ecdCD40L vector. CD8 T cells were isolated by CD4 depletion from the spleen cells of hMUC1.Tg mice 1 week after the completion of 2 subcutaneous injections (1 week apart) with the Ad-sig-ecdhMUC1/ecdCD40L vector (i) or from mice that were not vaccinated (ii). DCs that had been infected with the Ad-sig-ecdhMUC1/ecdCD40L vector were then mixed in a 1:1 ratio with the restimulated $CD8^+$ T cells. Proteins were isolated from these mixtures 0, 5, 15, and 45 minutes later and were separated using SDS-PAGE, transferred by Western blot analysis to a filter, and analyzed for phosphorylation of the p44 and p42 mitogen-activated kinase proteins using the New England BioLabs kit for phosphorylated proteins. The blot for the vaccinated mice is shown in panel i, and the blot for the unvaccinated mice is shown in panel ii.

injected 7 days earlier with the Ad-sig-ecdhMCU1/ecdCD40L vector and stimulated in vitro with the Ad-sig-ecdhMUC1/ecdCD40L vector–infected DCs.

CD8 T cells from unvaccinated hMUC1.Tg mice showed delayed kinetics and decreased total phosphorylation of ERK1 and ERK2 proteins (Figure 6Dii) compared with CD8 T cells from Ad-sig-ecdhMUC1/ecdCD40L–vaccinated hMUC1.Tg mice (Figure 6Di). These data suggest that Ad-sig-hMUC1/ecdCD40L vector injection induces an antigen-specific CD8 T-cell immune response to the MUC1 self-antigen through activation of the proliferation induction pathways in CD8 T cells.

Discussion

Our goal was to characterize the steps through which the vaccination of mice with the Ad-sig-TAA/ecdCD40L vector can induce an immune response to TAA-positive cells in anergic animals. Our experimental results suggest that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector leads to the continuous release of the TAA/ecdCD40L protein for at least a 10-day period. Binding of this protein to DCs induces increased levels of secondary signals of activation (CD80 and CD86) and the CCR-7 chemokine receptor on DCs, which lead to the migration of the TAA-loaded DCs to the regional lymph nodes. These events induce increases in the levels of the TAA-specific $CD8^+$ cytotoxic T lymphocytes in the spleens of Ad-sig-TAA/ecdCD40L vector–injected mice.

This increase in the TAA-specific $CD8^+$ lymphocytes in the Ad-sig-ecdhMUC1/ecdCD40L vector injected mice overcomes the anergy that exists to the hMUC1 antigen in hMUC1.Tg mice, which have expressed the hMUC1 antigen since birth. These experiments further show that inducing immunity is associated with the release of T_H1 cytokines, is HLA restricted, and is accompanied by an increase in the total phosphorylation of ERK1 and ERK2 pathways in T cells from vector-injected hMUC1.Tg mice when the T cells are exposed to Ad-sig-ecdhMUC1/ecdCD40L vector–infected DCs.

In contrast to the subcutaneous injection of the Ad-sig-ecdhMUC1/ecdCD40L vector, the subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce immune protection against the growth of the hMUC1-positive LL1/L2hMUC1 tumor cells (Figure 6A). This suggests that the danger signal[2] associated with the adenoviral vector carrying the ecdhMUC1/ecdCD40L transcription unit is an important part of overcoming the anergy to the hMUC1 antigen that exists in the hMUC1.Tg mice.

The oral TAA/CD40L *Salmonella typhimurium* DNA vaccine of Xiang and coworkers[14] had 3 potential limitations: the need for targeted IL-2 in addition to oral DNA bacterial vaccine; the use of a DNA vaccine that, because of its inefficiency of transfection, generated only low levels of expression for a short period of time; and the need to restrict the vaccination to the development of the antigen-loaded and activated DCs to the secondary lymphoid tissue of the gastrointestinal tract. Restriction to the T cells of the secondary lymphoid tissue of the gastrointestinal tract,[29] in accordance with the method of Xiang et al,[14] could be a limitation.

Because the adenoviral vector used in our work (and current results) can be administered to any part of the body, the homing of the T cells to the region of origin could be directed to the secondary lymphoid organs of any tissue by selection of the site of injection. In contrast to Xiang et al,[14] we found no need to follow up the vaccination of mice with targeted IL-2 treatment to break tolerance or to induce resistance to the engraftment of cancer cell lines in 100% of the vaccinated mice in our studies. Finally, we showed

that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is able to overcome the anergy that develops to TAAs, which are present from birth.

We had many reasons for selecting an in vivo method of activating and TAA loading DCs. The first is that our goal was to study the steps involved in the in vivo activation and antigen loading of DCs, not to compare in vivo and ex vivo loading of DCs. In vivo activation was an attractive option to study for several reasons. First, the work of Xiang et al[14] with the TAA/CD40L DNA vaccine involved in vivo vaccination, not ex vivo loading and activation. We wanted to determine whether we could improve on the in vivo activation and TAA loading seen when an adenoviral vaccine was used instead of a DNA vaccine. Second, in vivo activation by 1 or 2 subcutaneous injections of a vector could be vastly cheaper and simpler to administer than complex strategies involving ex vivo activation and TAA loading of DCs. Third, the in vitro activation approach was hampered by the limited number of DCs that could be produced, the inability to duplicate an in vivo environment in an in vitro culture system, and the short release as compared to the protracted in vivo TAA/CD40L protein release over a 10- to 14-day period when the ex vivo approach involved just a single injection. Finally, clinical trials involving ex vivo activation or tumor-antigen loading of DCs have proven to be less effective than in vivo methods of vaccination.[13]

A notable finding was that control experiments with vectors encoding TAA alone or CD40L alone were not as effective in activating DCs or inducing a cellular immune response against TAA-positive cancer cells in animal models. The question may be asked why the vaccination with vectors encoding the secretable fusion protein of the TAA/CD40L is more effective in inducing an immune response than vectors containing either TAA alone or CD40L alone. We have shown here that the chimeric TAA/CD40L fusion protein can form functional trimers, a requirement for binding the CD40L end of the fusion protein to the CD40 receptor on the DCs. Once the chimeric protein binds to the DCs, 2 things happen. DCs are activated to be effective at providing CD8 cells with the secondary signals necessary to activate CD8 TAA-specific T cells, and the chimeric TAA/CD40L protein is taken up into the DCs by endocytosis, thereby permitting the TAA to be processed in a way that results in its being available for presentation by MHC class I molecules. The fact that individual DCs are activated and TAA loaded is the advantage of the vectors encoding the TAA/CD40L fusion protein.

The immune response induced by the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is antigen specific and is dependent on the activation of the DCs in and around the vector injection site and on the migration of the TAA-loaded and activated DCs to the regional lymph nodes. It is not possible to overcome anergy with subcutaneous injection of the TAA/ecdCD40L protein or the subcutaneous injection of an adenoviral vector that carries a transcription unit encoding a nonsecretable TAA/ecdCD40L protein. These experimental results suggest that this approach to the activation of the immune response against tumor cells merits further study in preclinical and clinical models.

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